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The Chemotherapy of Calf Pneumonia
I. Some Pharmacologic Aspects of Sulfonamide Administration to Normal Calves

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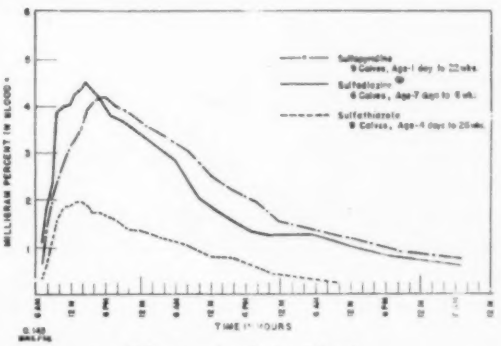
LITTLE INFORMATION is available relating to the pharmacology of sulfathiazole, sulfapyridine, or sulfadiazine in normal calves. Consequently, the experiments herein reported were carried out preliminary to the study of these sulfonamides in the treatment of calf pneumonia. Thorp *et al.*[1] administered sulfapyridine to 6 normal calves for five to six days in decreasing dosage. Three calves showed no bad effects but 3 exhibited toxic reactions, of which 1 died. Klein *et al.*[2] administered sulfapyridine to 6 cows to determine the optimum frequency of administration, resultant blood concentrations, and toxic effects. Stubbs *et al.*[3] administered sulfathiazole to 6 normal cows for eleven to twenty-four days and after one to two weeks noted the appearance of drug fever, leucopenia, and renal toxicity.

Normal calves were carefully chosen for these experiments. The calves were kept in individual pens and received the same feed, housing, and management as was normally provided at the University of Minnesota. All sulfonamide analyses were made on whole blood according to the method of Bratton and Marshall[4] with the aid of an Evelyn photometer. All samples were analyzed for free and total sulfonamide content.



*Mg. per 100 cc. of whole blood.

Fig. 1—The average blood concentration of free sulfapyridine, sulfadiazine, and sulfathiazole produced in normal calves by a single oral dose of 0.143 Gm./kg. of body weight.

Figure 1 summarizes the average blood concentrations of free sulfapyridine, sulfadiazine, and sulfathiazole produced by administering a single oral dose of 0.143 Gm./kg. (1 gr./lb.). Nearly 1,200 analyses were made on whole blood samples from 23 calves to produce these curves of sulfonamide concentrations. The average curves for total sulfonamide concentration in

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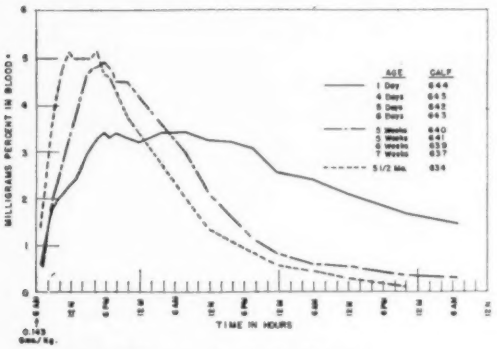
general paralleled the curves shown above; therefore they were omitted for greater ease in interpretation. For the first four hours after dosing, the blood concentration curves increased about the same for all three sulfonamides. This similarity indicated little varation in the respective rates



*Mg. per 100 cc. of whole blood.

Fig. 2—The influence of age on the average blood concentration of free sulfapyridine produced in normal calves by a single oral dose of 0.143 Gm./kg. of body weight.

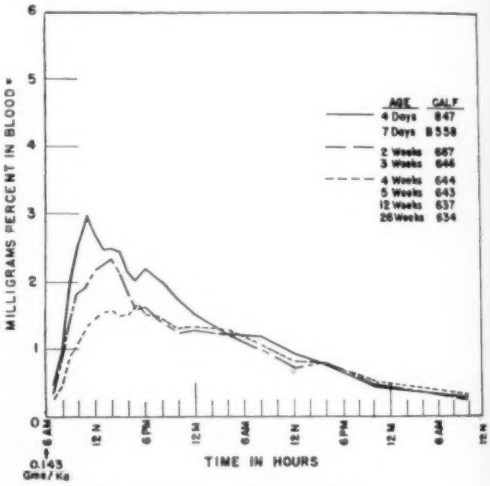
of absorption. Thereafter, the absorption of sulfapyridine lagged behind that of sulfadiazine as was shown by the blood concentrations. The maximum blood concentration of sulfathiazole attained was about one-half the level reached by the other two drugs. Proportionately, more sulfathiazole was required to produce a comparable blood level.

The sulfonamides did not maintain their peak concentration but decreased immediately, as would be expected. The rate of fall following the maximum concentration was 1 mg. per 100 cc. or less during a twelve-hour period. Free sulfathiazole was present in excess of 1 mg. per 100 cc. for only one day, whereas the blood concentration of sulfadiazine and sulfapyridine exceeded that level for about two and a half days.

INFLUENCE OF AGE ON BLOOD CONCENTRATIONS

The data reported in the preceding section were discussed as three comparable groups without regard to age. The data for each drug group were analyzed to determine whether the age of the calf influenced the rate of absorption, the maximum concentration, and the rate of disappearance of sulfonamides from the bloodstream. The results of analyzing the data for sulfapyridine are shown in figure 2.

Figure 2 shows that the 4 calves, 1 to 6 days of age, absorbed sulfapyridine from the gastrointestinal tract more slowly than the older calves because their blood concentrations rose more slowly. The maximum concentration was less than for the older calves by about 30 per cent; however, this maximum concentration was maintained for twenty-four to thirty-six hours instead of dropping rapidly as in the older calves, suggesting that absorption of sulfapyridine from the gastrointestinal tract of calves less than a week old extended over a longer period than in the older calves and was excreted much more slowly. The

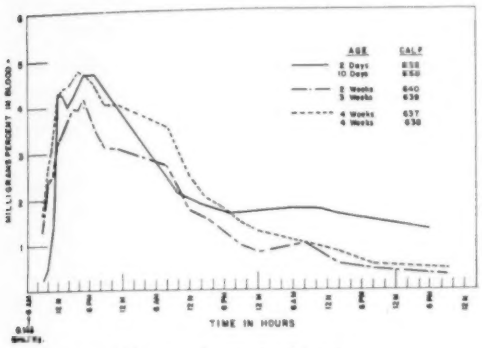


*Mg. per 100 cc. of whole blood.

Fig. 3—The influence of age on the average blood concentration of free sulfathiazole produced in normal calves by a single oral dose of 0.143 Gm./kg. of body weight.

rapid rise of blood concentrations of sulfapyridine in the older group of 4 calves, age 5 to 7 weeks, indicated a faster absorption and disappearance than in younger calves. Particular note was made of the persistence of the sulfapyridine in the bloodstream of the calves less than 1 week of

age. Three days after administration of a single oral dose of 0.143 Gm./kg. (1 gr./lb.) of body weight to each of 4 calves, their average concentration of free sulfapyridine was 1.5 mg. per 100 cc. of whole blood. The possibility of accumulation from repeated administration of sulfapyridine to newborn calves is great and also dangerous.



*Mg. per 100 cc. of whole blood.

Fig. 4—The influence of age on the average blood concentration of free sulfadiazine produced in normal calves by a single oral dose of 0.143 Gm./kg. of body weight.

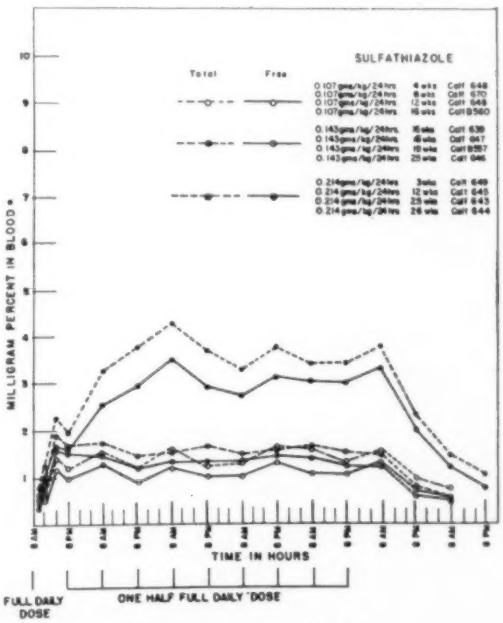
The third curve of figure 2 is for 1 calf, 5.5 months old and was only an indication of what may occur in older animals; namely, that both the absorption and disappearance of sulfapyridine are more rapid.

In figure 3 are data from three different age groups dosed with sulfathiazole. The rates of absorption of sulfathiazole, as indicated by blood concentrations, are the opposite of those of sulfapyridine. The younger calves immediately after dosing absorbed sulfathiazole more rapidly than the older ones. Following the administration of sulfathiazole, the 2 youngest calves, 4 days and 7 days old, showed higher rates of absorption as measured by the rate of increase in blood concentration during the first three or four hours than either age group of older calves. Four calves, 4 to 26 weeks of age, showed a slower rate of absorption, and the 2 calves, 2 and 3 weeks of age, were intermediate in their response.

The youngest calves receiving sulfathiazole also attained the highest blood concentration of drug, 3 mg. per 100 cc. of whole blood. The oldest calves, 4 to 26 weeks of age, attained a maximal blood concentration about one-half that of the youngest calves; those of intermediate age attained a level between the others. In contrast, the end results seem to be the reverse of those for sulfapyridine.

The effect of age differences on blood concentrations following a single dose of sulfathiazole soon disappear, so that in less than twenty-four hours after dosing the curves in figure 3 merge and decline together. Sulfathiazole did not persist in the bloodstream of any of the calves as did sulfapyridine.

Figure 4 shows the blood concentrations of sulfadiazine produced in 6 calves. No outstanding age differences were noted in a comparison of the blood concentration curves produced by the administration of



*Mg. per 100 cc. of whole blood.

Fig. 5—The average blood concentration of free and total sulfathiazole produced in normal calves at three levels of daily oral administration.

sulfadiazine. However, the ages of the calves did not vary as much as with the other two sulfonamides. Furthermore, only 6 calves were used as compared to 8 and 9 calves in each of the other experiments.

It seems likely that an age difference may exist for sulfadiazine just as for sulfathiazole and sulfapyridine but, if so, it is not as striking as for sulfapyridine.

It seems worthwhile to point out that the blood concentrations of sulfadiazine in the youngest calves, 2 and 10 days of age, persisted at a higher level than in the older calves. This would indicate a possibility of accumulation in the younger calf, as with sulfapyridine.

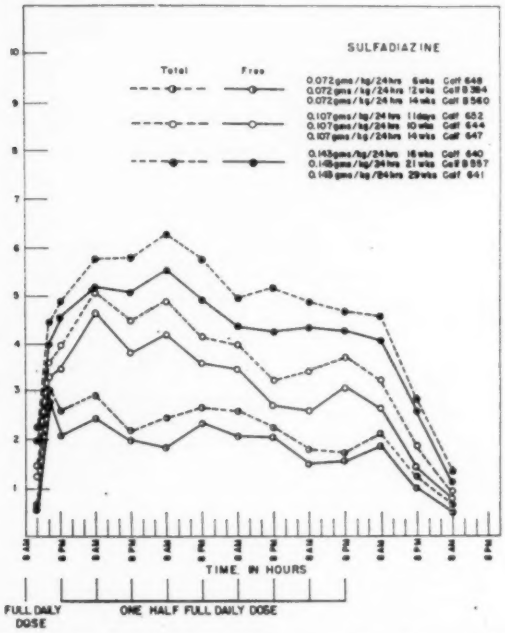
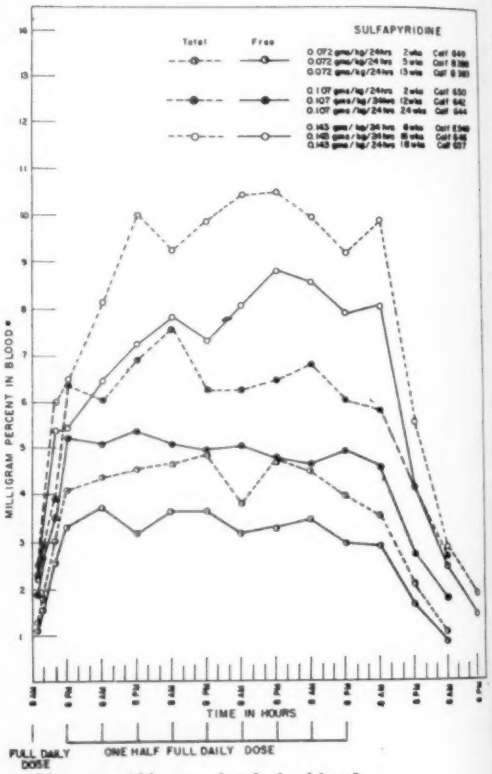


Fig. 6—The average blood concentration of free and total sulfadiazine produced in normal calves at three levels of daily oral administration.

AVERAGE BLOOD CONCENTRATIONS

These experiments were performed in part to determine what blood concentrations of sulfonamides would be produced in normal calves by daily oral administrations at three levels. They were intended to parallel practical farm conditions under which diseased calves would be treated routinely with sulfonamides. The calves used consumed normal amounts of fluids and dry rations. The first dose administered was a full daily dose. Every twelve hours thereafter, each calf received one-half of the full daily dose. The total dosing period was five days. The drugs were administered orally in capsules. To obtain the following data, over 1,200 analyses were made on blood samples from 27 different calves.

Figure 5 shows the average blood concentrations of sulfathiazole, both total and free, obtained at three different dosage levels. Four calves of varying ages received 0.107 Gm./kg. (0.75 gr./lb.) of sulfathiazole daily; another comparable group received 0.143 Gm./kg. (1 gr./lb.); and a third group received 0.214 Gm./kg. (1.5 gr./lb.). The average concentration curves for the first two groups indicate that only a slight difference exists between the

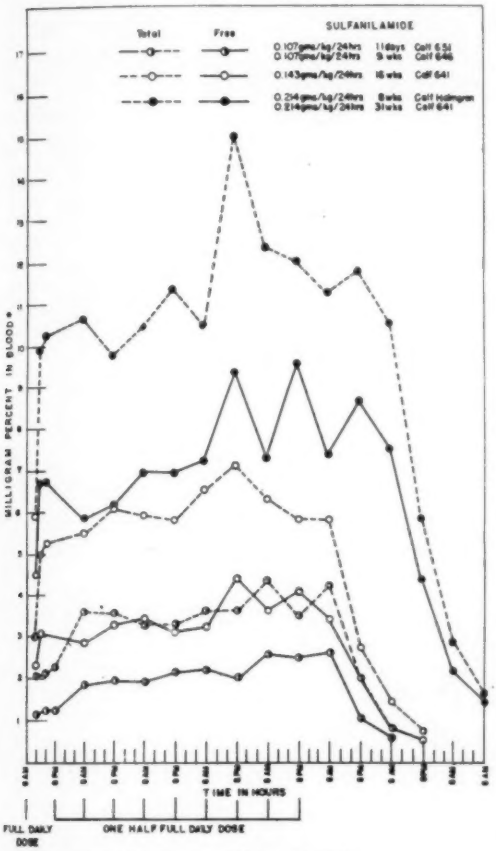


*Mg. per 100 cc. of whole blood.

Fig. 7—The average blood concentration of free and total sulfapyridine produced in normal calves at three levels of daily oral administration.

blood concentrations produced by 0.107 Gm./kg. and 0.143 Gm./kg. of sulfathiazole. The average blood concentration produced by 0.214 Gm./kg. of sulfathiazole was

roughly double that of the lower dosage levels. The average blood concentrations remained approximately the same after establishing a level and there appeared to be no tendency for accumulation of sulfathiazole in the tissues as shown by blood levels. These levels decreased markedly within twelve to twenty-four hours after the last dose and only traces remained thirty-six hours following the last dose.

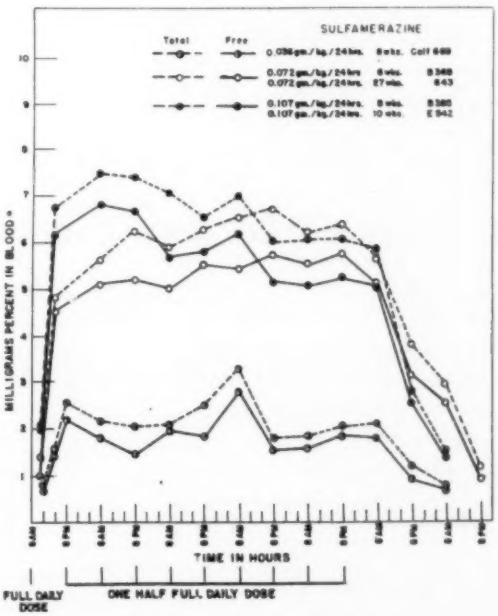


*Mg. per 100 cc. of whole blood.

Fig. 8—The average blood concentration of free and total sulfanilamide produced in normal calves at three levels of daily oral administration.

The above experiment was repeated using sulfadiazine at dosage levels of 0.072 Gm./kg. (0.5 gr./lb.), 0.107 Gm./kg. (0.75 gr./lb.), and 0.143 Gm./kg. (1 gr./lb.) of body weight. A total of 9 calves were used with 3 in each group. Figure 6 shows the average blood concentrations of free and total sulfadiazine produced at the above levels of dosing. The general blood concentration was considerably higher than that obtained with sulfathiazole, even though smaller amounts of sulfadiazine were administered. There was a proportionate spread between the blood concentration of sulfadiazine when correlated with the dosage level. There was no apparent danger of drug accumulation at any one of the above dosage levels during the experiment on the normal calves. The blood concentration began to decrease twelve to twenty-four hours after the last dose, and only small amounts of drug were present thirty-six hours after the last dose.

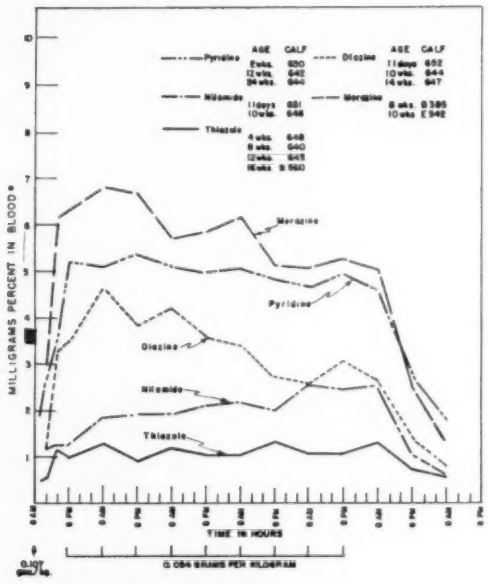
The same experiment was performed using sulfapyridine at the same dosage levels as for sulfadiazine: namely, 0.072, 0.107, and 0.143 Gm./kg. (0.5, 0.75, and 1.0 gr./lb., respectively) of body weight. A total of 9 calves were used in the experiment with 3 on each dosage level. The average blood concentrations of each group are shown in figure 7. The blood concentrations attain-



*Mg. per 100 cc. of whole blood.

Fig. 9—The average blood concentration of free and total sulfamerazine produced in normal calves at three levels of daily oral administration.

ed by sulfapyridine were higher than for sulfadiazine at the same dosage levels. There appeared to be considerable danger of drug accumulation at the dosage level of 0.143 Gm./kg. of body weight, as indicated by the progressively increasing blood concentration of sulfapyridine. Administration at this level appeared to be safe for



*Mg. per 100 cc. of whole blood.

Fig. 10—The average blood concentration of free sulfonamides produced in normal calves by the daily oral administration of 0.107 Gm./kg. of body weight.

about three days because accumulation was slight, but beyond three days the sulfapyridine accumulated significantly. The high blood concentration fell off rapidly from twelve to twenty-four hours after the last dose, although appreciable amounts were still present in the blood thirty-six hours following the last dose.

Some additional information was obtained with respect to sulfanilamide and sulfamerazine by using a limited number of calves to repeat the above experiments. Figure 8 shows the average blood concentrations obtained following the oral administration of sulfanilamide to 5 calves at levels of 0.107, 0.143, and 0.214 Gm./kg. (0.75, 1.0, and 1.5 gr./lb., respectively) of body weight. There would appear to be some danger of accumulation of sulfanilamide at the above dosage levels because all three of the average blood concentration curves show an upward trend. This is significant, however, only with the highest dosage level where possible toxicity might occur. The amount of total drug present in the blood is higher with sulfanilamide than any of the other compounds. The sulfanilamide in the blood of the calves at the two lower dosages was reduced to a small amount by thirty-six hours after the last dose, similarly to the other sulfonamides. However, seventy-two hours after the last dose, there was still an appreciable amount of sulfanilamide in the blood of the calves that received 0.214 Gm./kg. of body weight.

Figure 9 shows the average blood concentrations of sulfamerazine obtained in 5 calves by administering 0.036, 0.072, and 0.107 Gm./kg. (0.25, 0.5, and 0.75 gr./lb., respectively) of body weight. Relatively high blood concentrations are produced by small doses of sulfamerazine. Sulfamerazine did not accumulate at the above dosage levels, but it seemed to leave the blood

TABLE 1—Average Blood Concentrations Produced by Daily Oral Administration of Sulfonamides

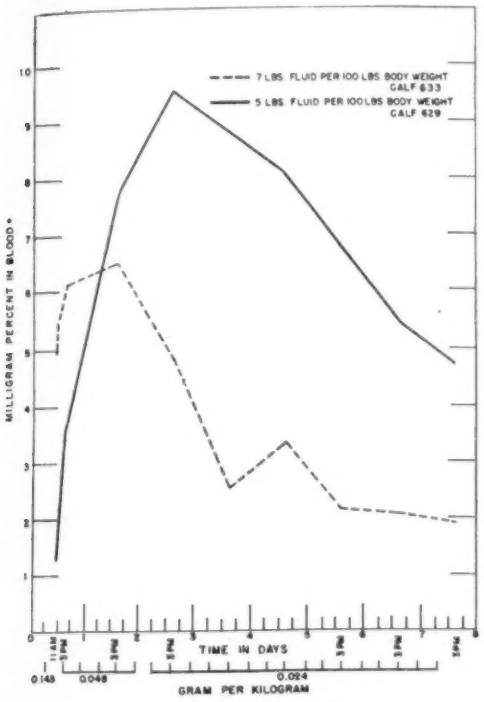
Drug	Gm./kg.	Approximate blood concentration in mg. per 100 cc. Free	Total
Sulfathiazole	0.107	1-1.5	1.25-1.75
	0.143	1.25-1.75	1.5 -1.75
	0.214	3-3.5	3.5-4
Sulfapyridine	0.072	3-3.75	3.75-4.75
	0.107	4.75-5.25	6-7.5
	0.143	6-Acc.	8-Acc.
Sulfadiazine	0.072	2-2.5	2.25-2.75
	0.107	3-4	3.5-4.5
	0.143	4.25-5.25	4.75-5.75
Sulfamerazine	0.036	1.5-2	1.75-2.5
	0.072	5-5.75	5.5-6.5
	0.107	5.5-6.5	6.25-7.25
Sulfanilamide	0.107	2-2.5	3.25-4.25
	0.143	3-4	5.5-6.5
	0.214	6-Acc.	10-Acc.

Acc. = accumulation.

0.036 Gm./kg. = 0.25 gr./lb.; 0.072 Gm./kg. = 0.5 gr./lb.; 0.107 Gm./kg. = 0.75 gr./lb.; 0.143 Gm./kg. = 1.0 gr./lb.; 0.214 Gm./kg. = 1.5 gr./lb.

stream slowly. At thirty-six hours after the last dose, appreciable amounts remained in the blood.

Figure 10 compares the average blood concentrations of five different sulfonamides obtained in each of the above experiments at the same dosage level of 0.107 Gm./kg. (0.75 gr./lb.) of body weight. The slowness in excretion of sulfapyridine is


7 LBS. FLUID PER 100 LBS. BODY WEIGHT CALF 633
5 LBS. FLUID PER 100 LBS. BODY WEIGHT CALF 629
MILLIGRAM PERCENT IN BLOOD*
TIME IN DAYS
0.143
0.048
0.024
GRAM PER KILOGRAM

*Mg. per 100 cc. of whole blood.

Fig. 11—The effect of fluid consumption on the blood concentration of free sulfapyridine.

worthy of note; sulfapyridine was present in highest concentration thirty-six hours after the last dose despite the fact that it did not attain the highest blood concentration during the experiment.

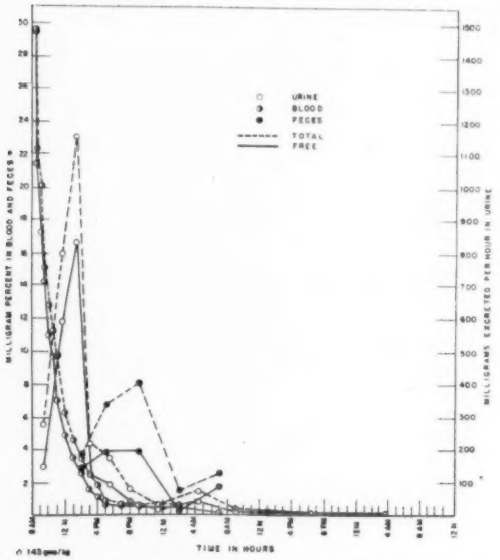
Table 1 summarizes figures 5 to 10 by giving the approximate range of blood concentrations that resulted from administering the stipulated amounts of drugs.

Accumulation of drug as marked by a progressive increase in blood concentration and the appearance of clinical toxicity reactions occurred with sulfapyridine at 0.143 Gm./kg. and with sulfanilamide at 0.214 Gm./kg. All the calves with high blood concentrations of sulfapyridine or sulfanilamide showed marked loss of appetite, depression, slight constipation, rough coat, and sunken eyeballs. These results would indicate that 0.107 Gm./kg. of sulfapyridine approaches the maximum safe dose for repeated administration to normal calves. If there is any variation for sick calves,

TABLE 2—Percentage of Acetylated Sulfonamides in the Blood and Urine of Normal Calves

Drug	Day of dosing	No. animals	% acetylation in blood	No. animals	% acetylation in urine
Sulfathiazole	1	12	21.47	4	36.22
	2	12	16.73	6	41.97
	3	12	20.20	5	49.08
	4	12	15.97	6	47.77
	5	12	19.33	4	41.50
	6	12	14.37	4	42.08
	7	12	18.77		
Average			18.12		43.103
Sulfadiazine	1	9	12.80	3	38.43
	2	9	11.40	3	42.10
	3	9	17.57	1	36.40
	4	9	14.73	3	35.97
	5	9	16.23	2	36.95
	6	9	13.57	2	27.45
	7	9	19.47		
Average			15.11		36.22
Sulfapyridine	1	9	18.13	2	45.50
	2	9	17.07	4	42.15
	3	9	23.17	2	46.20
	4	9	19.66	4	53.82
	5	9	22.60	1	62.50
	6	9	16.87	2	39.20
	7	9	19.17		
Average			19.52		48.23
Sulfanilamide	1	5	41.40		
	2	5	47.44	1	66.70
	3	5	38.52	1	68.10
	4	5	38.16	1	83.50
	5	5	32.14		
	6	5	37.18		
	7	5	30.02		
Average			37.84		72.77
Sulfamerazine	1	5	8.94		
	2	5	10.42	1	34.20
	3	5	17.92	1	40.00
	4	5	14.32	1	37.80
	5	5	13.50		
	6	5	13.14		
	7	5	13.62		
Average			13.12		37.33

their maximum safe dose would undoubtedly be less because of deranged metabolism. However, 0.143 Gm./kg. of sulfapyridine can be administered for a few days, not over three, if the dosage is then decreased to avoid further accumulation of drug. Accumulation with high dosage of sulfanilamide indicated that 0.214 Gm./kg. exceeded the maximum safe dose for prolonged administration. Again, however, this amount of drug can be given for three days with only a slight degree of accumulation, but thereafter the dosage must be decreased to avoid toxicity.



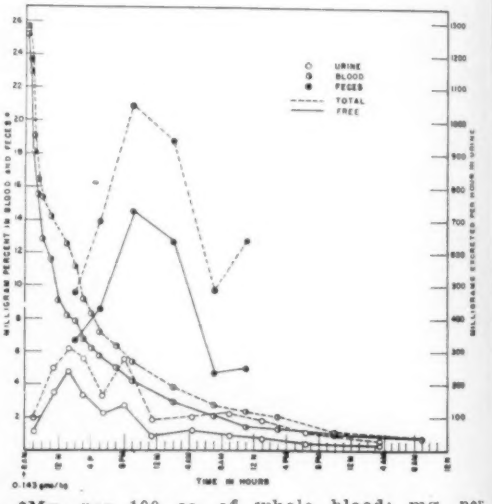
*Mg. per 100 cc. of whole blood; mg. per 100 Gm. of wet feces.

Fig. 12—The blood, urine, and fecal concentrations of free and total sulfathiazole produced by an intravenous injection of 0.143 Gm./kg. of sulfathiazole sodium.

These statements apply to calves with normal metabolism and consuming the usual amounts of fluid and feed. Clinical experience reported elsewhere[5] indicates that these findings must be applied cautiously to diseased calves because, in general, the sick animal does not tolerate high dosages of sulfonamides as well as the normal calf.

EFFECT OF FLUID CONSUMPTION ON THE BLOOD CONCENTRATIONS

A simple experiment was performed to demonstrate the influence of fluid consumption on sulfanomide blood concentrations in calves. The amounts of drugs and fluid administered were the same as might be used in treating a calf ill of pneumonia. Two calves were dosed with equivalent amounts of sulfapyridine for seven days: 0.143 Gm./kg. (1 gr./lb.) of body weight per day for the first two days and 0.072 Gm./kg. (0.5 gr./lb.) for the next five days. One calf received 7 lb. of fluid per 100 lb. of body weight, and the other calf received only 5 lb. of fluid per 100 lb. of body weight. Figure 11 demonstrates clearly that the calf on the lowest fluid intake had the highest blood concentration of sulfapyridine. The calf consuming approximately normal amount of fluid had blood concentrations of sulfapyridine within the expected range. It is interesting to note that the calf receiving the most fluid had higher initial blood concentrations of sulfapyridine than the other animal. This is possibly correlated with more extensive solution of drug in the larger amount of fluid ingested



*Mg. per 100 cc. of whole blood; mg. per 100 Gm. of wet feces.

Fig. 13—The blood, urine, and fecal concentrations of free and total sulfapyridine produced by an intravenous injection of 0.143 Gm./kg. of sulfapyridine sodium.

which resulted in more rapid absorption into the blood stream.

The above experiment was run simultaneously on 4 other calves, using sulfathiazole on 2 calves and sulfanilamide on 2 others. The results with each of these drugs paralleled, to a greater or lesser extent, the results noted above with sulfapyridine.

These experiments demonstrated the importance of proper fluid consumption during sulfonamide therapy in calves. Using only 6 calves in these three experiments does not remove the possibility of individual variation influencing the results. However, the results of each experiment are confirmed by those of the other two experiments. It appears that, if inadequate amounts of fluid are ingested, the drug accumulates in the body because there is not enough fluid to eliminate it by way of the urine. The sulfonamides are sparingly soluble in urine and, since they are excreted primarily by the urine, at least normal amounts of fluid should be ingested during sulfonamide therapy to insure proper elimination.

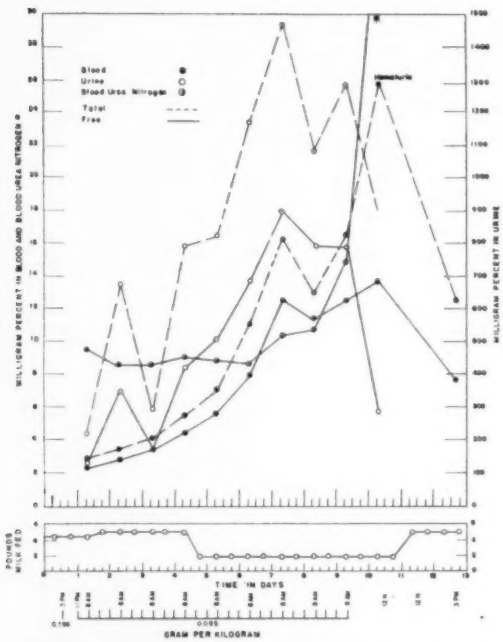
TABLE 3—The Excretion of Sulfonamides Administered Orally in Normal Calves

Drug Gm./kg.	Time	Range of mg. % free sulfonamide in blood†	Feces				Urine				Fluid intake cc. per day
			Gm. per day	Mg. %* sulfonamide Free	Mg. %* sulfonamide Total	Mg. sulfonamide Free	Mg. sulfonamide Total	Mg. % † sulfonamide Free	Mg. % † sulfonamide Total	Mg. sulfonamide Free	Mg. sulfonamide Total	cc. per day	
Sulfathiazole	11-26		1,362									1,100	3,462
	11-27		1,362									1,085	3,380
	11-28		1,498									1,000	2,326
0.572	11-29	1-6.3	1,407									920	3,335
0.214	11-30	6.3-8.7	1,401	6.48	6.84	91	95	516	834	5,263	8,506	1,020	2,735
0.214	12-1	8.5-9.6	681	14.36	18.88	98	128	348	621	3,828	6,831	1,100	4,333
0.214	12-2	9.6-8.3	1,498	19.00	24.00	285	360	298	561	3,789	7,152	1,275	2,427
0.214	12-3	6.8-5.6	1,136	21.81	28.36	246	321	304	624	3,222	6,614	1,060	4,583
94 Gm.	12-4	6.3-1.85	1,316	24.00	30.20	316	393	192	308	1,881	3,018	980	5,151
	12-5	1.3	1,362	23.80	24.80	324	337	134	220	1,340	2,200	1,000	
					Total....	1,360	1,634		.....	19,383	34,321	.....	
Sulfapyridine	11-26		1,135									1,190	3,576
	11-27		1,498									1,450	3,304
	11-28		1,634									1,150	2,214
0.429	11-29	1-6.65	635									1,050	3,304
0.143	11-30	6.65-10.9	2,406	5.60	8.64	134	206	124	204	1,972	3,243	1,590	1,851
0.143	12-1	10.9-9.35	1,270	14.00	15.36	178	186	402	648	3,738	6,026	930	3,440
0.143	12-2	9.35-8.8	1,634	14.07	21.80	228	355	472	795	3,776	6,360	800	2,305
0.143	12-3	7.3-7.2	1,634	13.30	21.54	216	350	388	648	3,337	5,573	860	1,305
56 Gm.	12-4	7.7-3.5	1,271	19.60	20.80	215	264	392	556	2,744	3,892	700	3,939
	12-5	1.85	1,589	12.70	14.20	202	226	187	298	2,711	3,321	1,450	
					Total....	1,173	1,587		.....	18,278	28,415	.....	
Sulfanilamide	11-26		1,180									1,140	4,470
	11-27		1,353									1,220	2,735
	11-28		1,725									1,180	4,107
0.572	11-29	1-6.45	1,907									1,160	4,697
0.214	11-30	6.45-11.4	1,498	4.44	4.76	66.6	71.4	140	348	2,072	5,150	1,480	2,200
0.214	12-1	11.7-10	1,362	10.88	13.44	147	182	320	630	2,680	5,670	900	2,881
0.214	12-2	9.6-81	2,860	11.60	29.36	332	841	393	500	2,554	3,250	650	2,654
0.214	12-3	8.6-6.5	1,407	12.40	23.40	174	328	368	564	3,312	4,876	900	3,744
100 Gm.	12-4	7.2-4.2	1,170	20.00	29.08	234	339	236	408	4,684	7,752	1,925	5,378
	12-5	3.19	1,044	10.35	11.20	107	118	200	348	2,700	4,698	1,350	
					Total....	1,061	1,879		.....	18,002	31,396	.....	
Control	11-26		1,135									815	3,122
	11-27		1,271									1,190	1,760
	11-28		1,226									1,180	2,431
	11-29		862									750	3,576
	11-30		1,316									630	2,259
	12-1		1,407									750	2,486
	12-2		1,498									820	3,349
	12-3		1,316									860	3,122
	12-4		1,362									1,200	3,031
	12-5		1,362									1,550	

*Mg. of sulfonamide per 100 Gm. of wet feces.
†Mg. of sulfonamide per 100 cc. of fluid.

ACETYLATED SULFONAMIDES IN BLOOD AND URINE

When a sulfonamide passes through the liver of most animals a part of it is acetylated, *i.e.*, an acetyl radical is added to the free amino group common to most sulfonamides. This acetylated compound, thereby, loses its antibacterial activity and in most cases becomes less soluble in the urine, so that the danger of urinary precipitation is increased. Thus, the degree to which a sulfonamide is acetylated becomes an important therapeutic consideration. The sulfonamides containing the pyrimidine ring are an exception to the above statement.



*Mg. of sulfathiazole and of blood urea nitrogen in 100 cc. of whole blood.

†B.U.N. increased progressively to 95.4 mg. per 100 cc. of whole blood or urine during the next five days followed by a decrease to 32.5 mg. per 100 cc. of whole blood or urine during the next three days.

Fig. 14—The influence of varied fluid consumption on the blood and urinary concentrations of free and total sulfathiazole and on the blood urea nitrogen. (Calf 620, weight 46.8 kilo., 10 weeks of age.)

The data from the multiple dose experiments on 27 calves mentioned above were analyzed to determine the percentage of acetylated sulfonamides in the blood and urine. The results are given in table 2. The data were analyzed also to determine whether the age of the calf or the different dosage levels of various drugs influenced the percentage of acetylation, but no significant differences were noted.

SULFONAMIDE EXCRETION DATA

Excretion data for sulfathiazole, sulfapyridine, and sulfanilamide are presented in table 3 for 3 normal calves and 1 control calf. One drug was administered to each calf every eight hours at the indicated dosage levels, and all feces and urine were collected for a week. These observations were quantitative in nature but, unfortunately, were terminated too soon. Termination of the experiment was not altogether by choice because it was practically impossible to keep the crude apparatus harnessed onto the calves and in place after collecting samples for 10 days.

TABLE 4—Summary of Elimination of Sulfonamides Administered Orally

Drug	Total drug administered (Gm.)	Total drug excreted in urine (Gm.)	Total drug excreted in feces (Gm.)	Total drug recovered	
				(Gm.)	%
Sulfathiazole	94	34.32	1.63	35.95	38.1
Sulfapyridine	56	28.42	1.59	30.01	53.6
Sulfanilamide	100	31.40	1.88	34.28	34.3

The data of table 3 are summarized in table 4. These data are incomplete because of the early termination of the experiment and are included only as an indication of what may be expected in the early phases of such an experiment.

Four experiments were performed to follow the disappearance of sulfapyridine and sulfathiazole from the bloodstream and its excretion into the urine and the intestinal contents. A single dose 0.143 Gm./kg. (1 gr./lb.) of each drug as the sodium salt was injected intravenously into 2 dif-

ferent calves so that a total of 4 animals was used. Frequent blood and urine samples were analyzed for sulfonamide content. Urinary excretion of sulfonamides could be expressed quantitatively because total urinary excretion was measured.

The two experiments on each drug were confirmatory. The data from one experiment with each drug were selected to plot in graphic form as shown in figures 12 and 13. These figures show the rapid decrease in the sulfathiazole blood concentration from nearly 30 mg. per 100 cc. of whole blood to 1 mg. per 100 cc. of whole blood during the first eight hours. During the same period, the sulfapyridine blood level decreased from 25 to about 6 mg. per 100 cc. of whole blood. The more rapid disappearance of sulfathiazole than of sulfapyridine is explained to considerable extent by noting the comparative urinary excretions during the first eleven hours following the intravenous injection of drug. Table 5 shows that sulfathiazole is excreted into the urine two to three times as rapidly as sulfapyridine during the first eleven hours following the intravenous administration. Figures 12 and 13 show that the maximum level of urinary excretion of sulfathiazole in mg. per hour was nearly four times the maximum level for sulfapyridine. However, by forty-nine hours after the intravenous administration of drugs, the percentage of the total dose of sulfapyridine excreted in the urine was nearly as much as for sulfathiazole. From these data, it would appear that sulfathiazole is excreted much more readily by the kidney of the calf than is sulfapyridine, but that the total urinary excretion of both drugs is approximately the same two days after single intravenous doses.

More sulfapyridine was excreted into the feces than was expected following intravenous administration of the drug. The analyses were not quantitative but a maximal concentration of 21 mg. per 100 Gm. of wet feces was reached in feces passed about twelve hours after the drug was administered. Much smaller concentrations of sulfathiazole were found in the feces which could be expected in view of the rapid decrease in the blood concentrations of sulfathiazole.

About two-fifths of the sulfathiazole and sulfapyridine injected intravenously into the 4 calves was unaccounted for by urinary excretion at the end of the experiment. Some of the drug which had been taken up by body tissues probably was released slowly into the urine and feces after the experiment terminated. It is possible that limited amounts of these drugs may be metabolized by the tissues to form degradation products not recognized when excreted.

SULFATHIAZOLE TOXICITY

Calf 620 received 0.286 Gm./kg. (2 gr./lb.) of sulfathiazole daily for nine and a half days. In addition, the fluid intake was reduced to 40 per cent of the normal consumption. Figure 14 shows the results of this treatment. The total blood concentration of sulfonamide reached a value of 25.8 mg. per 100 cc. of whole blood and was accompanied by a severe hematuria and other signs of urinary involvement. At the same time, the blood urea nitrogen increased until it reached a maximum of 95.4 mg. per 100 cc. of fluid. The urinary

TABLE 5—The Urinary Excretion of Sulfathiazole and Sulfapyridine

Drug	Animal	11 hr. after injection of drug		49 hr. after injection of drug	
		Total urinary excretion (Gm.)	% of total dose*	Total urinary excretion (Gm.)	% of total dose*
Sulfathiazole	644	4.741	52.8	5.138	57.2
	646	4.535	57.5	4.844	61.3
Sulfapyridine	645	2.268	25.3	5.103	57.0
	Holm.	1.215	18.7	3.579	54.7

*Corrections were made for the weight of the sodium and water of crystallization present in the sulfathiazole sodium sesquihydrate and the sulfapyridine sodium monohydrate that was injected intravenously.

excretions of sulfonamide paralleled the increasing blood concentrations until the tenth day of the experiment when the total urinary concentration showed a sharp decline, in spite of the excessive increase in the total blood concentration of sulfonamide on that day. This decrease in kidney activity was reflected by a coincidental increase of the blood urea nitrogen by two and a half times. The nervous derangement, apparently a peripheral neuritis as judged by clinical symptoms, appeared suddenly following the occurrence of the excessive drug concentrations in the blood. Table 6 gives the detailed data including the clinical symptomatology. After a week of drug administration the numbers of leucocytes increased during the height of the toxic reaction with an increased percentage of granulocytes. There was an over-all decrease in the hemoglobin and red blood cells. Calf 620 survived this experiment to grow into an apparently healthy, but undersized, yearling heifer.

TABLE 6—Protocol of Calf 620 — Effects of Excessive Concentrations of Sulfathiazole

	Dosage	Blood in mg.*		Urine in mg. %*		Feces in mg. %*		Hb.	RBC	Lymph. PMN		BUN
Date	Gm./kg.	Free	Total	Free	Total	Free	Total	(Gm.)	(1,000's)	WBC	% Seg.%	mg.%
4-28	0.286											
4-29	0.286	2.6	3.0	137	227	13.8	22.5	11.0	6,590	6,800	85 15	9.7
4-30	0.286	2.9	3.6	364	684	6.4	7.8	9.4	5,800	7,600	84 15	8.7
5-1	0.286	3.5	4.2	172	308	29.6	48.3					8.7
5-2	0.286	4.5	5.6	424	792	25.7	34.9					9.2
5-3	0.286	5.7	7.05	512	828	62.7	87.5					
5-4	0.286	8.5	11.2	684	1,176	48.0	72.5	10.4	5,970	9,150	80 20	8.7
5-5	0.286	12.7	16.4	908	1,472	51.0	68.0					10.5
5-6	0.286	11.5	13.1	804	1,088	38.7	51.1					10.8
5-7	0.286	12.7	16.6	792	1,288	60.0	75.0					15.0
5-8		13.8	25.8	284	896	27.0	41.2	11.7	6,630	10,650	60 40	37.9
5-10		7.7	12.5									
5-11												92.5
5-12								9.7	5,750	5,700	75 25	95.4
5-15												32.5
5-20								7.7	4,390	6,250	69 30	

5-8 —hematuria; frequent urination; depression.
5-9 —unable to stand; weakness in hind legs; lies on sternum.
5-10—lies on side; hypersensitiveness; legs rigidly extended; milk force fed.
5-11—only slight hypersensitiveness; lifted to feet; legs bent and back arched; apparent pain; heart action continued strong.
5-12—much improved; gait slightly stilted; gained 5 lb. in 2 weeks.
5-13—much better; active.
5-15—acts normally.
*Mg. per 100 cc. of whole blood or urine; mg. per 100 Gm. of wet feces.

SUMMARY

1) Nearly 1,200 analyses of blood samples from 23 normal calves were made to establish the curves of average blood concentrations resulting from single oral doses of sulfathiazole, sulfapyridine, and sulfadiazine.

2) Sulfathiazole, sulfapyridine, and sulfadiazine were absorbed in normal calves at about the same rate for the first four hours after dosing; thereafter, sulfapyridine was absorbed somewhat slower than sulfadiazine. The rates of absorption varied somewhat with the age of the calf.

3) A given dose of sulfathiazole attained only about one-half the blood concentration of similar doses of sulfapyridine and sulfadiazine.

4) At a given single dosage, sulfapyridine and sulfadiazine persisted in excess of 1 mg. per 100 cc. of whole blood for about two and a half times as long as sulfathiazole in normal calves. The tendency for sulfapyridine and sulfadiazine to persist was exaggerated in newborn calves.

5) Over 1,200 analyses of blood samples from 27 different normal calves were made to establish the curves of average blood concentrations resulting from various dosage levels of sulfathiazole, sulfapyridine, sulfamerazine, and sulfanilamide administered for five successive days.

6) There was dangerous accumulation of sulfapyridine and sulfanilamide when administered for more than three days at dosages of 0.143 Gm./kg. (1 gr./lb.) and 0.214 Gm./kg. (1.5 gr./lb.), respectively. There was danger of accumulation of sulfapyridine at all ages, but it was more pronounced in calves under 1 week or age.

7) The percentage amounts of acetylated sulfonamides in the blood of normal calves dosed for five days could be arranged in the following increasing order: sulfamerazine, sulfadiazine, sulfathiazole, sulfapyridine, and sulfanilamide.

8) Clinical signs of toxicity, accompanied by an accumulation of drug in the blood stream, were: marked loss of appetite, depression, slight constipation, rough coat, and sunken eyeballs. Additional signs of advanced drug toxicity were hematuria, renal colic, oliguria or anuria, and peripheral neuritis in extreme sulfathiazole toxicity.

9) A decrease in fluid consumption caused a marked increase in the blood concentration of sulfonamide as compared to the normal.

10) A decrease in fluid consumption resulted in a delayed absorption period as measured by the appearance of sulfonamides in the blood stream.

11) The maximum rate of urinary excretion of sulfathiazole following an intravenous injection was nearly four times as fast as for sulfapyridine. However, the total amount of sulfapyridine excreted within forty-nine hours after the intravenous injection nearly equaled the amount of sulfathiazole excreted. Approximately 60 per cent of the administered drugs was recovered within the first forty-nine hours after administration. No quantitative measure was made of the drug excreted in the feces, which was a minor factor in the total elimination.

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The Chemotherapy of Calf Pneumonia
II. The Use of Sulfathiazole, Sulfapyridine, and Sulfadiazine in the Treatment of Calf Pneumonia

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BY 1942 when this study was initiated, sulfanilamide had been used by many veterinary practitioners in treating calf pneumonia and other diseases. The opinion generally held, but infrequently reported in literature, was that sulfanilamide aided in the recovery of some cases of calf pneumonia but by no means all cases. Gibbons[5] reported that sulfanilamide had reduced the mortality of calf pneumonia by 25 per cent. He also mentioned that sulfapyridine had been used only on a few cases but gave a better response than sulfanilamide. Thorp[11] reported the recovery of 44 out of 47 cases of calf pneumonia treated with sulfapyridine. Later, Thorp[12] reported the recovery of 11 out of 12 cases treated with sulfadiazine and 31 out of 33 cases treated with sulfathiazole. However, all the diseased animals presented were treated and no control data were available to permit an accurate evaluation of the benefits of the sulfonamide therapy.

The toxic effects of sulfonamides in calves have not been frequently noted. Farquharson[4] used sulfapyridine and sulfathiazole with good results in treating calf diphtheria. The most severe toxic reaction noted was hematuria in 3 cases treated with sulfapyridine; no such reaction was observed in those calves treated with sulfathiazole. No particular changes were noted in the blood constituents during the first few days of therapy, although a leucopenia was observed in several animals after five to six days of treatment. Thorp *et al.*[13] reported the occurrence of calculi in the renal pelvis and hemorrhage and casts in the renal tubules of 1 case treated with sulfapyridine. Clinical signs of toxicity were observed in 2 other calves including anemia and leucocytosis. Klein *et al.*[7] observed mild symptoms of toxicity in normal cows after several days' administration of sulfapyridine, *i.e.*, leucocytosis with increased per cent of neutrophils, urticaria, anorexia, and decreased milk flow. Stubbs *et al.*[10] noted mild toxic reactions after several days of sulfathiazole administration to normal cows, including neutrophilia, drug fever, urticaria, and hypersensitiveness of some areas. One cow died. She showed gross and microscopic kidney lesions accompanied by renal concretions. Hepatic and splenic degeneration were also noted.

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MATERIALS AND METHODS

1) *Sources of Material.*—Clinical cases of calf pneumonia were secured from the following sources.

University of Minnesota	
Dairy calves	18 cases
Beef calves	4 cases
Experimental dairy calves purchased from farmers	5 cases

TABLE I—Clinical Cases of Calf Pneumonia Treated with Sulfathiazole

Case No.	Animal			Drug		Days of Therapy		Mgs. % Non-acelylated Sulfathiazole§		Termination
	Age in Weeks	Sex	Weight in lbs.	Gms./Kg.	gr./lb.	Each period	Total	Range	Average	
4	6	F	130	0.214	1.5	3	3	2.7-4.5	3.22	R
7	24	S	350	0.191	1.33	3	3	3-3.5	3.15	R
8	28	F	400	0.214	1.5	3.5	3.5	2.5-3.5	2.66	R
9	28	S	400	0.214	1.5	2	2	2-2.5	2.43	R
15	4	F	60	0.191	1.33	2.5		1.7-2.7		
				0.286	2	2	4.5	1.2-3	2.1	D
16	5	M	113	0.214	1.5	3	3	2-4.5	2.27	D
18	4	M	92	0.286	2	4	4	3.5-5-5	4.9	R
20 †	4	M	98	0.286 *	2	4		1-2		
				0.429	3	5	9	2-5.5	2.36	D
28	10	F	150	0.286	2	2		2.7-3.2		
				0.143	1	2	4	2.5-3	2.9	R
29	4	F	94	0.214 ‡	1.5	2		1.5-1.7		
				0.286	2	4	6	2-7	2.92	R
31	10	F	128	0.214 ‡	1.5	2		1-2		
				0.286	2	2		1.5-3.5		
				0.358	2.5	3	7	6-19	6.74	D
36	12	F	160	0.286	2	5	5	2.5-6.5	3.65	R
38	10	F	130	0.286	2	4	4	2-4.2	2.96	R
47	3	F	75	0.286	2	4	4	2.5-4	3.6	R
52	5	F	126	0.286	2	3	3	2.5-5.7	3.65	R
57	3	F	123	0.286 *	2	3	3	4-6	4.48	R
62	12	F	180	0.143 *	1	3.5	3.5	1-2	1.33	R
67	11	F	180	0.143 *	1	3.5	3.5	1.5-2.5	1.9	R
72	8	M	145	0.143 *	1	5	5	1-3	1.51	R
84	20	F	400	0.214	1.5	1.5		1.5-2		
				0.143	1	1	2.5	0.75-1.5	1.24	R
92	8	M	155	0.214	1.5	7	7	3-4	3.6	R
97	4	F	110	0.143	1	4.5	4.5	1.5-2	1.75	R
101	2	M	85	0.214	1.5	4	4	2.5-4	3.45	R
104	4	M	130	0.143	1	3	3	2-4	2.65	R
108	1	M	60	0.214	1.5	3	3	2-3	2.65	R

*Initial therapy included 0.072 Gm./kg. of sulfathiazole sodium injected intravenously.
†Clinical relapse.
‡Initial therapy included 0.143 Gm./kg. of sulfathiazole sodium injected intravenously.
§Mg. per 100 cc. of whole blood.

Farm Security Administration
Dairy calves .....10 cases
Stockyards' sources ..... 6 cases
Dairy and beef calves owned by dairy farmers .....52 cases

Total number of clinical cases ..95

It is believed that the clinical cases constitute a representative cross section of the cases of calf pneumonia found in the average midwestern veterinary practice. If there was any variation from the average practice, the diseased calves were more ill than ordinarily encountered. The relatively large number of milk producers in the milk shed of the St. Paul-Minneapolis area increased the number of advanced cases of calf pneumonia received because many of these men were primarily interested in the milk production of the cows and had neither time nor quarters to care for newborn calves.

2) *Housing, Care, and Feeding.*—All of the sick calves received the same feed, housing, and management as normal calves maintained at the University of Minnesota. An individual pen was provided for each calf. Primarily, one isolated building was used to house the clinical cases, although overflow cases were placed in another building similarly constructed. Desired temperatures and ventilation were readily maintained in these structures.

3) *Methods.*—All sulfonamide analyses were made on whole blood according to the method of Bratton and Marshall,[3] using an Evelyn photometer. The same procedures were followed as were employed in analyzing the samples in the studies on normal calves[4].

4) *Experimental Procedure.*—When a suspected case of calf pneumonia was available, the patient was thoroughly examined. When making a diagnosis, history, auscultation of

TABLE 2—Clinical Cases of Calf Pneumonia Treated with Sulfapyridine

Case No.	Animal: Age wks.	Sex	Wt. lb.	Drug: Gm./kg.	gr./lb.	Days of therapy: Each period	Total	Mg. % ‡nonacetylated sulfapyridine: Range	Av.	Termination
1†	10	M	150	0.214	1.5	4		9-12		
				0.143	1	5	9	6-7.5	8.1	D
2	16	M	274	0.214	1.5	3	3	8-12	9.96	R
13†	6	F	70	0.214	1.5	4.5				
				0.143	1	1.5	6	6-15		
				0.214	1.5	2.5	2.5	10-14	9.91	D
17	3	M	77	0.143*	1	3	3	7-10	8.6	D
19	6	M	111	0.143	1	4	4	7-13	10.34	R
22	3	M	80	0.143	1	3	3	6-10	7.72	R
35	4	F	90	0.143	1	2		5.5-7.5		
				0.107	0.75	3	5	6.5-7.5	6.66	R
39	12	F	150	0.143	1	2.5	2.5	6.5-8	7.1	R
40	10	F	110	0.179	1.25	3	3	7-7.5	7.2	R
42	2	F	60	0.214*	1.5	3		9.5-10.5		
				0.143	1	2		10-10.75		
				0.107	0.75	2	7	10.5-15	10.83	D
45	16	F	200	0.214*	1.5	2	2	5-7	5.9	D
50†	2	F	93	0.143	1	4	8	4.6-10	7.41	D
				0.143	1	4				
55	16	F	186	0.143	1	2		7-8		
				0.072	0.5	3.5	5.5	3-5	5.52	R
60	3	F	130	0.143	1	3.5	3.5	6.75-7.75	7.15	R
65	3	M	115	0.143	1	1.5		5-6		
				0.095	0.66	4	5.5	2-4	4.51	R
70	3	F	110	0.143	1	3	3	6-7	6.2	R
76	14	M	160	0.143	1	2		7-8		
				0.072	0.5	3		2.5-4		
				0.143	1	3.5	8.5	4.5-6	4.8	R
79	8	F	160	0.143	1	3	3	5-6	5.6	R
83	3	F	77	0.143*	1	4		4-8		
				0.107	0.75	3	7	6-8	5.32	R
87	2	F	85	0.143*	1	4		3.5-7		
				0.107	0.75	2	6	10-10.5	6.74	R
91	5	M	150	0.143	1	3	3	5-7	6.1	R
95	4	M	138	0.143	1	4	4	5-8.5	6.8	R
99	2	F	95	0.143	1	3		6.5-8		
				0.095	0.66	2	5	4-5	5.9	R
105	2	F	106	0.143	1	2		6-8		
				0.095	0.66	2	4	4-5.5	5.8	R

*Initial therapy included 0.072 Gm./kg. of sulfapyridine sodium injected intravenously.
†Clinical relapse.
‡Mg. per 100 cc. of whole blood.

the thorax, temperature, pulse, and respiration were emphasized but not to the exclusion of other considerations such as pressure on the trachea to provoke coughing, percussion of the thorax, and the general clinical condition of the calf.

As positive cases of calf pneumonia were received for treatment, they were placed by rotation in four different groups according to the therapy instituted: (1) sulfathiazole, (2) sulfapyridine, (3) sulfadiazine, and (4) untreated controls. An exception to this procedure occurred during the treatment of the first 20 cases which were divided into three groups, to the exclusion of sulfadiazine therapy. The untreated controls received the same housing, care, and ration as the drug-treated patients until death or recovery.

The first dose of drug was a full daily dose because of the need of saturating the body tissues and establishing a satisfactory drug concentration immediately. The initial large dose was followed at proper intervals by divided doses. All of the drugs were administered orally in gelatin capsules. Calves with acute pneumonic involvement and showing symptoms of severe pulmonary edema received as initial treatment an intravenous injection of the sodium salt of the sulfonamide employed. About half of the calculated daily dose was administered intravenously as the sodium salt in warm, sterile, distilled water

TABLE 3—Clinical Cases of Calf Pneumonia Treated with Sulfadiazine

Animal				Drug		Days of therapy		Mg. % *nonacetylated sulfadiazine		
Case No.	Age wks.	Sex	Wt. lb.	Gm./kg.	gr./lb.	Each period	Total	Range	Av.	Termination
44	4	M	105	0.143	1	2.5	2.5	4.5	4.5	D
46	3	M	90	0.143	1	8	8	5-6	5.6	R
49†	6	F	131	0.143	1	1.5				
				0.095	0.66	3	4.5	4.5-5.5		
				0.143	1	1.5				
				0.095	0.66	3	4.5	4.5-6.5	4.86	D
51	2	F	90	0.143	1	2	2	4-6	5.2	D
54†	3	F	92	0.143	1	1.5		6.5-10.5		
				0.095	0.66	3	4.5	6-7		
				0.143	1	1.5		5.5-7.5		
				0.095	0.66	5.5	7	2.5-4	5.7	R
56	1	M	70	0.143	1	7	7	6-10	7.9	R
59†	9	M	135	0.143	1	4.5	4.5	4-7		
				0.143	1	2				
				0.095	0.66	4.5	6.5	3-3.5		
				0.143	1	3		3-4.5		
				0.095	0.66	7	10	2-3	2.8	R
61	2	M	75	0.172	1.2	2		5-6		
				0.086	0.6	8	10	2-3	2.84	R
64	10	F	140	0 143	1	1.5		3-4		
				0.095	0.66	3	4.5	3-4	3.45	R
66	10	F	140	0.143	1	1.5		5-6		
				0.095	0.66	4	5.5	2.5-3.5	4.84	R
69	2	M	97	0.143	1	1.5				
				0.095	0.66	3	4.5	3-5	4.2	R
71	1	F	67	0.143	1	2.5	2.5	3.3-12.7	6.55	D
74	2	M	70	0.143	1	1.5		5-5.6		
				0.107	0.75	2	3.5	5-6	5.31	R
77	2	F	79	0.143	1	3	3	4-6.5	5.57	R
80†	2	F	97	0 143	1	3		7-8		
				0.143	1	4.5	7.5		7.5	R
82	9	F	135	0.143	1	3		5-7		
				0.072	0.5	1.5	4.5	5-6	5.7	R
85	12	F	160	0.286	2	2.5		13.5		
				0.143	1	2	4.5	21	17.0	R
88	2	M	75	0.143	1	3		3-5		
				0.214	1.5	5	8	10-20	7.75	R
90	3	F	90	0 214	1.5	1.5		13-15		
				0.143	1	3.5	5	15-17	15.7	R
96	24	M	275	0.214	1.5	5	5	9-13	4.33	D
100	2	F	40	0.143	1	1.5				
				0.095	0.66	2.5	4	2-5	3.65	R
103†	3	M	75	0.214	1.5	1.5		9-14		
				0.143	1	2		8-9		
				0.086	0.6	4	7.5	2.5-3		
				0.214	1.5	2.5				
				0.143	1	8	10.5	3-6	5.44	R
107	6	F	140	0.143	1	3		5-7		
				0.095	0.66	2	5	4-6	5.1	R
109	2	M	115	0.143	1	3		5.5-6.5		
				0.095	0.66	3	6	4-5.5	5.2	R

*Mg. per 100 cc. of whole blood.
†Clinical relapse.

or 10 per cent dextrose; the remainder of the calculated daily dose was given orally as the sulfonamide. The first of the divided doses was administered at the next dosing period, eight or twelve hours after the initial dose.

TABLE 4—Untreated Cases of Calf Pneumonia

The untreated cases that recovered were 5, 30, 37, 43, 68, 89, and 106. The untreated cases that died were 10, 23, 41, 48, 53, 58, 63, 73, 75, 78, 81, 86, 94, 98, and 102.

A minimum of one blood sample a day was collected during the therapy period from all clinical cases, with only occasional exceptions. Efforts were made to collect urine and fecal specimens, but the samples were irregular and limited in number. Nevertheless, some data derived from urine and fecal samples are presented later.

Patients that improved in general health and condition were observed for one to five weeks before being discharged. Calves that responded well to treatment were generally kept under observation for seven to ten days after the disappearance of fever, depression, abnormal pulse and respiration, and inappetance. However, subsequent inquiries and, if possible, examinations were made of the patient during the next four to six weeks. Some discharged patients were readily available and were examined frequently. A special effort was made to check for cases relapsing following treatment, and several such patients were identified. It is possible that some patients suffered reinfections, but it seemed more probable that relapses occurred, especially in the more chronic infections, because of the nature of the etiologic agent and the lesion it produced.

Dosages employed were based upon the results of sulfonamide administration to normal calves. However, a few of the earlier cases were treated while the initial pharmacologic studies were in progress and, as a result, received larger doses than were necessary.

RESULTS FROM TREATMENT OF CLINICAL CASES

The data were gathered from naturally occurring, clinical cases of calf pneumonia. All cases that revealed complicating conditions either during the course of treatment or on autopsy have been omitted.

Tables 1, 2, and 3 summarize the results of the experimental treatment of clinical cases of calf pneumonia with sulfathiazole, sulfapyridine, and sulfadiazine. Table 4 records the termination of the disease in a group of untreated controls, and table 5 gives a brief summary of the previous tables. All of the animals were treated with divided dosage at twelve-hour intervals except for a few of the early cases receiving sulfathiazole and sulfapyridine, which were treated at eight-hour intervals. The data from the pharmacologic experiments[6] and from the initial clinical cases indicated that administration of the drugs at twelve-hour intervals produced a fairly uniform and probably adequate blood level throughout the 24-hour period. Generally, the twelve-hour dosing interval was used because it was thought to be adequate and more adaptable to the routine of calf husbandry in effect.

Average Duration of Therapy.—Table 6 summarizes the average duration of therapy. Sulfonamides were administered until the patients no longer showed signs of respiratory infection. When drug therapy was stopped, the calves had not returned to normal condition but signs of

TABLE 5—A Summary of the Clinical Cases of Calf Pneumonia

Treatment	Total no. cases	Termination					
		Recoveries	(%)	Av. mg. %* nonacetylated sulfonamide	Deaths	(%)	Av. mg. %* nonacetylated sulfonamide
Sulfathiazole	25	21	84.0	2.88	4	16.0	3.37
Sulfapyridine	24	18	75.0	6.63	6	25.0	8.46
Sulfadiazine	24	19	79.2	6.38	5	20.8	5.09
Untreated controls	22	7	31.8		15	68.2	
Total cases	95						

*Mg. per 100 cc. of whole blood.

pneumonia were absent and the patients were definitely on the way to recovery. Table 6 shows that it was necessary to treat diseased calves somewhat longer with sulfadiazine than with sulfathiazole and sulfapyridine to bring them to the same stage of clinical recovery. Patients that relapsed and required more than one course of treatment were excluded from this summary.

TABLE 6—Average Duration of Therapy Excluding Patients Suffering Relapses (in days)

Drug	Recoveries	Deaths
Sulfathiazole	3.83	4.83
Sulfapyridine	4.31	4.0
Sulfadiazine	5.66	3.0

Clinical Relapses.—Some of the calves with pneumonia responded and appeared to be recovering satisfactorily until suddenly they showed a severe relapse. Only one relapse occurred among the patients treated with sulfathiazole, while three relapses occurred with sulfapyridine and five with sulfadiazine. Thus, it would appear that relapses are more frequent with sulfadiazine and less frequent with sulfathiazole.

Hematologic Data.—In the beginning, frequent blood samples were studied during the treatment of many patients. Later, only the initial and final blood samples were examined hematologically. This was discontinued when no significant variation was observed between the initial and final counts. The main object in examining the blood samples was to note any abnormality of major clinical significance, such as anemia and agranulocytosis, that might develop as a result of the sulfonamide therapy. No such conditions were noted in the animals studied that could be clearly attributed to the sulfonamide therapy.

Data was presented from only 4 cases treated with sulfadiazine, so little can be concluded regarding the effects of this drug on the blood picture. The higher leucocyte count for sulfadiazine shown in table 8 may be accounted for largely by 1 case out of the 4 having a marked leucocytosis. Fluctuations in the white cell count were due largely to reciprocal variations in the polymorphonuclear cells and the lymphocytes. Little or no variation was noted in other leucocytes.

Excretion of Sulfonamides in Clinical Cases.—Excretion data and the corresponding blood concentrations of 26 clinical cases treated with sulfonamides are presented in table 9. The data are arranged by the day within each drug group with minimum, maximum, and average values given for each type of analysis. The values are all given in mg. per cent, inasmuch as it was impracticable and, in most cases, impossible to secure quantitative measurements. These data were intended to reveal the maximum concentration of excreted sulfonamide attained in the urine and feces of representative clinical cases during therapy. There was no intention of following the concentrations of drug to the end of the excretion period. The maximum concentrations produced during the excretion of clinical dosages of sulfonamides are significant, inasmuch as these concentrations approach the maximal solubility of the sulfonamides in urine beyond which renal concretions are formed with serious complications.

TABLE 7—Summary of Relapses in Clinical Cases

Drug	No. relapses	% total cases	Termination	
			Recoveries	Deaths
Sulfadiazine	5	20.8	4	1
Sulfapyridine	3	12.5	0	3
Sulfathiazole	1	4.0	0	1

TABLE 8—Summary of Hematologic Data of Clinical Cases

Sulfa	No. cases	Count	Average of all cases			
			Hhb (Gm.)	RBC (1,000's)	WBC	PMN (%)
thiazole	12	Initial	10.9	6,030	7,171	22.4
		Final	11.0	5,928	8,041	35.4
pyridine	12	Initial	10.9	5,985	7,119	37.8
		Final	10.6	5,717	8,973	41.4
diazine	4	Initial	11.3	7,799	7,688	40.2
		Final	12.5	6,878	11,186	46.4

TABLE 9—Excretion of Sulfonamides in Clinical Cases

Sulfa	Dosage Gm./kg.	No. cases	Dosing day	Blood concentration in mg. %*				Urinary concentration in mg. %*				Fecal concentration in mg. %†			
				Free		Total		Free		Total		Free		Total	
				Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.	Range	Av.
thiazole	0.286	10	2	0.75-5.07	2.26	0.97-6.8	2.76	71-672	356.6	135-1104	521.1	2.4-23.2	12.0	4.8-25.8	15.3
	0.286	10	3	0.8-5.77	2.75	1.1-7.32	3.65	64-564	352.7	109-1512	635.8	6.7-44.7	25.3	8.8-87.8	32.0
	0.286	10	4	1.4-6.15	3.27	1.7-6.8	3.92	105.2-704	393.9	157.9-1200	650.9	8.9-53.7	38.5	17.7-60.1	46.3
	0.286	8	5	1.7-5.8	3.64	2.4-6.2	4.33	161.7-685	496.4	251.7-1160	764.6	19-49.7	37.2	39.2-84.3	55.8
	0.286	3	6	4.3-6.5	5.07	4.9-7.3	5.77	180-520	392	232-868	616	22.9-88	47.3	27.4-105	60.8
pyridine	0.143	10	2	4.1-7.55	5.99	5.05-11.2	7.86	18.4-218	127.6	29-600	332.7	5.5-49.5	17.8	10.5-52.6	24.5
	0.143	10	3	5.4-10.05	7.62	7.8-13.1	10.35	25.6-452	176.6	76-792	344.3	7.5-60	18.7	15.9-165	42.5
	0.143	9	4	5.6-12.5	8.12	7.1-15	10.51	32-344	166.8	99-620	341.8	7.4-26.4	16.8	19.9-45	32.0
	0.143	3	5	6.4-7.0	6.77	7.7-8.6	8.20	264-396	334.7	368-628	538.7	15.3-114.5	49.4	21.6-142	63.2
	0.143	3	6	6.5-10.1	7.87	7.9-11.9	9.33	164-244	209.3	292-456	400	4.5-56	33.2	8.3-94	59.9
diazine	0.143	6	2	1.3-5.25	3.33	1.5-6.8	4.04	41.4-62.4	53.6	100-217	157.4	3.7-57.4	23.7	7.1-68.4	29.2
	0.143	6	3	5.6-9.4	7.03	7.45-11.35	10.53	61-109	76.1	188-367	266.7	13.5-47.2	26.6	21-52.1	32.9
	0.143	5	4	6.2-13	9.03	9.35-15.25	11.46	49.6-109.6	77.84	119-387	229.1	19.3-54	39.2	20.5-57.7	42.4
	0.143	2	5	6.4-9.2	7.8	7.7-13.8	10.75	52.3-52.5	52.4	172-217	194.5	42.2-49.5	48.9	47.9-75	61.5
	0.143	2	6	10.75-12.6	11.7	15.7	15.7	50.4-55.2	52.8	107-197	152	110-217	163.5	131.241	186.3

*Mg. per 100 cc. of whole blood or urine.
†Mg. per 100 Gm. of wet feces.

The blood concentrations produced by these drugs are given in table 9 to permit correlation with the excretion data.

Percentage of Acetylated Sulfonamide in Blood, Urine, and Feces of Clinical Cases.—The percentage of acetylated sulfonamide present in the blood, urine, and feces of treated clinical cases is shown in table 10. All of the calves in each drug group were on the same dosage, although for varying lengths of time. The figures presented in table 10 are averages of several cases within each drug group.

TABLE 10—Per Cent Acetylation of Sulfonamide in Clinical Cases

Sulfa	No. cases	Dosage Gm./kg.	Day*	% acetylation Blood	Urine	Feces
pyridine	10	0.143	2nd	22.65	54.50	34.41
	10	0.143	3rd	25.77	51.81	50.40
	9	0.143	4th	22.73	52.18	43.20
	5	0.143	5th	19.30	42.37	29.65
	4	0.143	6th	18.70	42.22	47.60
Average				21.83	48.62	41.05
diazine	8	0.143	2nd	17.14	66.92	23.48
	8	0.143	3rd	21.85	72.64	18.90
	6	0.143	4th	23.91	64.13	18.15
	4	0.143	5th	20.35	75.90	28.10
	3	0.143	6th	20.60	59.20	13.05
Average				20.77	67.56	20.34
thiazole	10	0.286	2nd	26.90	34.85	21.45
	10	0.286	3rd	24.47	40.55	23.31
	10	0.286	4th	17.09	38.99	19.81
	8	0.286	5th	17.42	34.80	33.41
	4	0.286	6th	14.42	36.95	22.10
Average				20.06	37.23	24.02

*Day of dosing.

Blood Urea Nitrogen Concentrations in the Clinical Cases.—Numerous blood urea nitrogen analyses were made on blood samples from clinical cases in an effort to follow the progress of the sulfonamide therapy and particularly to anticipate the appearance of symptoms of renal toxicity. In general, the increase in blood urea nitrogen did not appear earlier than the first clinical symptoms of toxicity were apparent to a careful observer, so the determinations were of less value than had been hoped.

The data in table 11 present the results of analyzing 112 blood samples drawn from 31 clinical cases during therapy for deter-

TABLE II—The Blood Urea Nitrogen Concentrations in Treated Cases of Calf Pneumonia

Drug	Case no.	Total blood sulfonamide in Mgm. % †	Blood Urea Nitrogen in Mgm. % †			Termination
			Range	Average	Analyses	
Sulfathiazole	16	2.1-3.3				
		4.2-5.9	23.4	23.4	1	D
	20	4.75-6.9	14.5-20.5	17.2	3	D
	28	3.6-4.2	12.8-14.8	13.8	2	R
	29	1.7-2.3	5.0-7.7	6.5	3	
		2.5-8.0	8.5	8.5	1	R
	31	1.3-4.5	5.5-11.7	8.4	4	
		6.7-14.5	6.3-7.3	6.8	2	
		17.3-37.0	25.5-32.3	28.8	2	D*
	36	3.1-7.3	6.2-15.7	10.0	3	R
	38	2.4-4.6	14.5-16.2	15.3	2	R
	47	3.3-4.8	6.2	6.2	1	R
	52	2.8	12.2	12.2	1	
		3.5-7.3	13.7-88.0	38.6	3	R*
	57	5.0-6.6	7.2-8.3	7.8	4	R
	84	1.9-2.7	6.45	6.45	1	
			18.3	18.3	1	R
Sulfapyridine	17	11.0-14.0	32.5	32.5	1	D
	22	9.0-13.0	6.3-12.2	9.8	3	R
	35	6.3-8.7	5.2-11.7	8.45	2	R
	39	8.0-10.0	6.2-12.7	9.45	2	R
	50	9.0-13.5	7.8-14.3	11.4	7	D
	55	8.0-11.0	24.5	24.5	1	
		3.5-6.0	14.5-17.5	16.0	2	R
	60	9.5-10.6	10.5-14.5	12.1	4	R
	76	9.0-10.0	19.0	19.0	1	
		2.8-4.8	9.5-15.5	13.4	3	
		5.3-7.5	16.0	16.0	1	R
	83	5.3-10.0	7.2-16.5	10.6	5	R
	87	4.5-8.3	4.5-6.7	5.5	4	
		12.0-14.0	10.7	10.7	1	R
Sulfadiazine	49	5.1-7.6	7.0-8.0	7.5	2	D
	56	3.0	7.0	7.0	2	
		6.5-15.7	12.0-27.5	16.2	9	R
	59	3.4-5.1	6.0	6.0	1	R
	71	1.7-4.2	15.7-16.5	16.1	2	
		9.0-14.7	17.7-18.7	18.2	2	D
	74	6.8-9.35	14.7-17.7	16.3	4	R*
	77	5.05	14.5	14.5	1	
		7.45-9.6	18.5-21.3	19.8	3	R*
	80	2.05	13.8	13.8	1	
		7.7-10.6	20.5-24.7	22.6	4	R*
	82	5.9	14.0	14.0	1	
		9.4	23.5	23.5	1	R
	85	15.6	15.8	15.8	1	
		25.1	53.2	53.2	1	R
	96	1.9-2.65	9.6-14.3	12.3	3	
		3.0-3.65	19.3-26.7	23.0	2	
		4.7-18.2	39.0-88.0	59.1	4	R

*Clinical signs of drug toxicity in urinary system *i.e.*, hematuria, frequent urination with straining, oliguria, gelation of urine, anuria.
†Mg. per 100 cc. of whole blood.

TABLE 12—Protocol of Case 31 Showing the Occurrence of Fatal Renal Block with Sulfathiazole

Date	Dosage Gm./kg.	Blood mg. %* Free	Blood mg. %* Total	Urine mg. %* Free	Urine mg. %* Total	Feces mg. %* Free	Feces mg. %* Total	Hb. (Gm.)	RBC (1,000's)	WBC	Lymph (%)	PMN Seg. %	BUN Mg. %*	Fluid/ day cc./kg.
5-13	0.232	1.8	2.5							3,850	65	35	10.0	45.4
5-14	0.232	1.2	1.4	71.0	135	6.2	8.4						11.7	45.4
5-15	0.286	0.8	1.1	136.0	268	70.5	87.8	8.6	4,920	4,800	93	7	6.5	45.4
5-16	0.286	1.4	1.7	388.0	576	45.8	58.0							45.4
5-17	0.286	3.5	4.5	604.0	928	44.8	53.8						5.5	4.54
5-18	0.358	6.1	6.7	788.0	1,164	120.0	133.0	8.6	4,900	4,750	96	4		45.4
5-19	0.358	13.5	14.3	1,076.0	1,480	125.0	146.0	8.9	5,630	5,450	93	7	6.3	45.4
5-20		13.2	17.3	148.0	268	147.0	201.0	10.7	6,460	9,100	60	40	7.3	60.5
5-21		19.5	37.0	42.4	80	150.0	207.0						32.3	60.5
5-22	Death													

Clinical symptoms of drug toxicity: 5-19—trace of hematuria. 5-20—severe hematuria; severe depression; gelation of urine; nervousness. 5-21—peripheral paralysis; anuria.

Gross pathology of urinary tract: kidneys edematous and twice normal size; precipitated sulfonamide crystals in renal tubules.

*Mg. per 100 cc. of whole blood or urine; mg. per 100 Gm. of wet feces.

minations of the blood urea nitrogen content. The total sulfonamide content of the blood is also given in order that it may be correlated with the urea nitrogen concentrations.

In most of the patients, the blood urea nitrogen did not exceed normal limits during sulfonamide therapy. In a few patients showing definite clinical signs of urinary blockage, the blood urea nitrogen levels attained abnormally high concentrations. In some cases, high blood urea nitrogen concentrations apparently occurred from the disturbance of body metabolism existing during severe pneumonia. This complication plus other possible factors greatly limited the value of the blood urea nitrogen determination as an indication of renal disturbances arising from sulfonamide therapy. In fact, only in the presence of clinical signs of drug toxicities was a high blood urea nitrogen level considered to be due to drug precipitates. This limitation reduced the blood urea nitrogen determination to the level of a confirmatory test rather than a means of anticipating renal toxicities before the clinical signs appeared.

Drug Toxicities.—In administering sulfonamides to calves, few toxicity reactions were noted. The calves in this investigation were treated for an average of four to five days, rarely more than seven days, which may account for the absence of minor toxic reactions attributable to prolonged therapy. The skin and temperature were not altered by the sulfonamides, and no major blood changes were consistently noted. The chief drug toxicity in calves following sulfonamide administration was the inability of the urinary system to excrete large amounts of drug with subsequent stoppage of urinary excretion. This condition was a grave portent because there was little that could be done to save the life of the patient beyond increasing fluid consumption. Urinary function was carefully noted in all of the patients.

The first clinical signs of toxicity in the calves observed following the administra-

tion of sulfathiazole, sulfapyridine, or sulfadiazine were a mild depression and inappetance. Also, the coat was rough; the eyes were dull and sunken; excreted drug adhered to the vulvar or preputial hair; temperature, respiration, and heart action were not affected. The calves urinated frequently and strained in an effort to void urine. The reaction occurred every minute or so in some cases. It was particularly noticeable in the heifer calves because of the humping of the back as part of the urination reflex. Usually, samples of urine voided in such manner contained from traces to readily discernible amounts of blood. Almost invariably, a nervousness accompanied the repeated efforts to urinate. The calf was uneasy, restless, and continually moved about the pen. It was characteristic to see a calf walking somewhat aimlessly around the pen but stopping every few feet to void a few drops of urine. If the depression became more severe the animal spent most of the time lying listlessly on the floor. The blood urea nitrogen levels sometimes were five to seven times the concentrations existing when treatment was begun. The inappetance by this time amounted to a complete refusal of feed and of practically all fluid. Urine either was not voided or small amounts were passed from a recumbent position. The calf in this state had to be forced to its feet and often assisted in order to maintain its position. The hind quarters were weak and even wobbly.

A clinical drug toxicity peculiar to sulfathiazole was noted where an excessive dosage level was employed that brought the animal rapidly to a toxic condition. The condition may be clinically described as a peripheral neuritis. A calf receiving an excessive amount of sulfathiazole and in the advanced stages of toxicity described above developed a hypersensitiveness to touch followed by an inability of the legs to raise the body. The legs could still be moved voluntarily but would not lift the body from the floor. As the condition progressed there was a complete loss of leg function so the calf could no longer rest on its sternum but had to lie on its side. The legs were somewhat stiffly extended and the head and neck were drawn up and backwards. Occasionally, purposeless running movements and slight convulsions were observed. One such case of acute sulfathiazole toxicity was produced. Clinical case 31 resulted from accidental overdosing with sulfathiazole and terminated fatally within two days of the appearance of the symptoms.

TABLE 13—Cases Showing Clinical Signs of Renal Toxicities

Drug	Case no.	Termination
Sulfapyridine	42	Death
	45	Death
Sulfathiazole	31	Death
	52	Recovery
Sulfadiazine	74	Recovery
	77	Recovery
	85	Recovery

The hyperesthesia and the sciatic paralysis with some brachial involvement closely resembles the paralysis reported in chickens by Bieter *et al.*[2], who demonstrated the toxic action of six sulfonamides on nervous tissue, especially of the peripheral nerves. Cases of peripheral neuritis in man have been reported following the administration of sulfapyridine, sulfathiazole, sulfadiazine, and sulfanilamide[3].

TABLE 14—A Summary of the Autopsy Findings of the Treated Fatalities

Sulfa	No. fatalities	No. cases with drug concretions in urinary system	Cases showing only lesions of pneumonia: No. cases	Cases showing only lesions of pneumonia: % total cases treated
pyridine	6	2	4	16.6
diazine	5*	0	5	20.8
thiazole	4	2†	2	8.0

*One case not subjected to necropsy but showed no clinical signs of urinary distress.
†One case accidentally overdosed; only one kidney of the second case contained drug concretions.

Data from case 31 are presented in table 12 to demonstrate the correlation of over-

dosage, excessive blood and urine concentrations of sulfonamide, the subsequent malfunction of the urinary system, and the postmortem findings of edematous kidneys containing drug concretions. The increase in blood urea nitrogen following the appearance of clinical signs of toxicity seemed to be slower and smaller than in other patients.

TABLE 15—Incidence of Pulmonary Abscesses in the Fatalities

Drug	No. autopsies	No. cases with extensive pulmonary abscesses
Sulfapyridine	6	2
Sulfadiazine	5*	2
Sulfathiazole	4	3
Untreated	15	4

*One case not subjected to necropsy.

Clinical cases of calf pneumonia that showed signs of renal toxicity during treatment are listed in table 13. All three drugs were effective in treating prolonged chronic cases, but the calves were in better physical condition and suffered less weight loss when treated with sulfadiazine.

Autopsy of the Fatalities among the Clinical Cases.—Postmortem examination of the 15 fatalities from the group of 22 untreated calves confirmed the previous diagnoses of calf pneumonia. Several of these cases occurred as acute processes that subsided into chronic pneumonia and pleurisy and soon died. In some cases, the initial acute bronchopneumonia progressed rapidly to a fatal termination.

Fatalities among the treated cases were carefully examined at necropsy with two major objectives: (1) to examine the urinary system for the presence of drug crystals or concretions, and (2) to examine the respiratory system and correlate the findings with the clinical observations. A few cases showed lesions of gastroenteritis in addition to pneumonia and were eliminated from this experimental study.

All the cases subjected to necropsy showed extensive lesions of pneumonia, thus verifying the previous diagnoses. A summary of the necropsy findings of the treated fatalities is given in table 14.

There were nearly as many treated fatalities with abscesses in the lungs as were found among the untreated fatalities. Apparently pneumonic tissues containing abscesses do not yield well to treatment with the above three sulfonamides. It was clinically apparent that several of the cases referred to in table 15 did not respond to therapy. These cases seemed to be caused by low grade infections that produced a chronic type of calf pneumonia refractory to sulfonamide therapy.

Bacteriologic Findings of All Fatalities.—Thirty experimental calves which died during the investigation were examined at necropsy. Several tissues from each calf were cultured bacteriologically. An effort was made to culture the tissues as soon as practicable following death of the animal. Standard techniques and mediums were employed in the culturing, including mediums containing citrates and hemolyzed whole blood. The bacteriologic findings are given in table 16.

The bacteriologic findings were variable. Six different organisms were isolated, all of which have been reported as being isolated from clinical cases of calf pneumonia. It was not surprising that tissues of the 3 calves dying from renal blockage were sterile because the bacteriostatic action of the high blood levels of the sulfonamide plus the action of the natural antibodies of the patient probably overcame the pathogenic microörganisms, leaving the tissues sterile on bacteriologic culture.

It would appear that one organism is not more susceptible to the sulfonamide therapy than another bacterium, but that the difference in clinical response may depend upon other factors, such as the time the sulfonamide therapy is instituted in the course of the disease and the occurrence of pulmonary abscesses, which would protect and maintain the infective agent. As indicated in table 16, abscesses were found associated with *Pseudomonas aeru-*

ginosa in 4 cases, *Pasteurella multocida* in 3 cases, *Corynebacterium pyogenes* in 3 cases, and with a nonhemolytic Streptococcus in 1 case. It seemed odd that no abscesses were found in conjunction with the 4 cases of *Staphylococcus aureus*.

All six species of pathogenic bacteria were isolated also from the untreated fatalities of calf pneumonia. Three fatalities among the untreated controls were free of pathogenic bacteria although, unfortunately, scant growths of a saphrophytic contaminant appeared in cultures from 2 of the calves. Cultures of the tissues from the third fatality, case 10, were repeatedly sterile on culture. An effort was made to explain the absence of a bacterial pathogen in the tissues of case 10 by searching for a virus agent.

Case 10 presented a peracute calf pneumonia characterized by rapidly progressing pulmonary edema. Immediately upon death of the animal, the entire respiratory tract was removed, cultured, and frozen at –20 F. Experiments aimed at the reproduction of the disease in young calves by a possible virus agent were performed, using the lungs of case 10 as a source of infective material. Nine normal calves were inoculated intranasally and intratracheally with infective material. Four calves did not respond. Five calves, 2 receiving coarse Berkefeld filtrate and 3 receiving lung tissue suspension, responded, but the symptoms did not resemble those of typical calf pneumonia and the results were discounted at the time. The symptomatology noted was transient in nature and consisted of: suddenly appearing diarrhea and dyspnea lasting for three to eighteen hours; an increase in temperature to 103.5 to 104 F.; and a general weakness and poor condition with occasional coughs. The entire condition was brief and somewhat indefinite when compared to the calf pneumonia generally encountered. A calf inoculated intranasally and intratracheally with a suspension of sterile, normal lung tissue gave

TABLE 16—Bacteriologic Findings of All Fatalities

Sulfa	Case no.	Bacterial pathogen	Complicating organism
thiazole	15*	*Pasteurella multocida*	
	16*	*Pseudomonas aeruginosa*	
	20*	*Pseudomonas aeruginosa*	
	31	Sterile†	
pyridine	1	*Staphylococcus aureus*	*Escherichia coli*
	13*	*Corynebacterium pyogenes*	
	17	*Pasteurella multocida*	
	42	Sterile†	
	45	Sterile†	
	50*	Non-hemolytic Streptococcus	
diazine	44	Sterile	
	49	*Escherichia coli*	
	51*	*Corynebacterium pyogenes*	
	71	No necropsy	
	96*	*Pasteurella multocida*	
Untreated	10	Sterile	
	23	Sterile‡	
	41*	*Pasteurella multocida*	
	48	*Pasteurella multocida*	
	53	*Corynebacterium pyogenes*	Hemolytic Streptococcus
	58	*Pasteurella multocida*	
	63*	*Corynebacterium pyogenes*	Hemolytic Streptococcus
	73	*Staphylococcus aureus*	*Escherichia coli*
	75	*Staphylococcus aureus*	
	78	Hemolytic Streptococcus	
	81*	*Pseudomonas aeruginosa*	
	86	Sterile‡	
	94	*Escherichia coli*	
	98	*Staphylococcus aureus*	
	102*	*Pseudomonas aeruginosa*	

*Extensive abscess formation in lungs noted at necropsy.

†Renal concretions of sulfonamides resulted in the death of 2 patients and were judged to be the primary etiologic factor in the third patient.

‡Scant growths of a nonpathogenic contaminant appeared in two cultures.

no response. One of the calves that received the Berkefeld filtrate was presented for necropsy at the close of its brief clinical response, and examination of the lungs revealed a few isolated lobules of congestion and early consolidation. These tissues were frozen but deteriorated when the refrigerating unit failed, thus killing any virus present. The full significance of these findings was not appreciated until later when Baker[1] published his full report on the virus agent of calf pneumonia and described the same symptomatology which was observed in the above brief experiments. Although no serial transmission experiments were performed, there is reason to believe that the symptomatology produced in the calves inoculated with Berkefeld filtrate and the tissue suspension was caused by the virus agent of calf pneumonia and enteritis described by Baker.

DISCUSSION

Table 17 contains average data computed from results of the clinical experiments to serve as a possible guide in the administration of sulfathiazole, sulfapyridine, and sulfadiazine in the treatment of calf pneumonia.

TABLE 17—A Maximum Dosage Schedule for Calves up to 4 Months of Age, Based upon Data Obtained from the Clinical Cases

Sulfa	Dosage/day				Free sulfonamide in mg. %*	
	Gm./kg.	gr./lb.	Divided doses/24 hr.	Days of dosing	Expected range	Av. range
T	0.214	1.5	2-3	2-3	1-4.5	2-3.34
	0.143	1.0	2-3	1-3	0.75-3	1.45-2.2
P	0.143	1.0	2	1-2	5-10	6-8
	0.095	0.66	2	2-3	3-7	4-6
D	0.143	1.0	2	2-3	3-10	5-7
	0.095	0.66	2	2-5	2-7	3.25-4.6

T= thiazole. P= pyridine. D= diazine.
*Mg. per 100 cc. of whole blood.

Pneumonia infrequently attacks calves under 2 weeks of age. During this investigation, only 3 patients treated were less than 2 weeks old. However, it was apparent that newborn calves should not be dosed quite as heavily as older calves and that they should be observed closely during treatment for signs of toxicity. The kidneys of the newborn calf do not seem to excrete sulfonamides as effectively as those of the older calf.

Early in the use of sulfonamides in human beings, the importance of maintaining a proper fluid intake and urinary volume was recognized as being necessary for proper drug excretion and avoidance of urinary complications[9]. Similarly, it became apparent early in this investigation that the maintenance of normal fluid intake in the calf was fundamental to successful sulfonamide therapy. If inadequate amounts of fluid were ingested and excreted, the point of maximum solubility of a sulfonamide would be exceeded in the urine, and sulfonamide crystals would precipitate in the urinary system, chiefly the renal tubules. Patients in which appreciable renal concretions had developed gave little or no response to treatment and died immediately. Calves treated for calf pneumonia with sulfonamides should be given the normal amounts of fluid. If the fluid is not voluntarily ingested, manual administration by stomach tube is important. When the first clinical signs of drug toxicity are noted, the forcing of fluids is indicated in an effort to promote the renal excretion of the sulfonamides and to correct the tendency for drug precipitation in the urinary tract.

Judging from the clinical responses in this investigation it is important to begin therapy as early as possible in the course of the disease. Early in an infection, there are proportionately many more leucocytes and immune bodies to combat the bacterial pathogens. These antibody mechanisms are necessary to kill the susceptible pathogens which are only inhibited from multiplying and probably not destroyed by the sulfonamides. Delay in instituting treatment means a decrease in the natural disease barriers of an animal and a prolonged re-

covery or perhaps even death. Furthermore, it is known that exudates and tissue necrosis neutralize the action of sulfonamides so treatment should be instituted before these processes become extensive enough to interfere appreciably with the drug action. Early treatment of calf pneumonia is also important in those cases which, though susceptible in the early stages, soon subside into a refractory stage of chronicity characterized by pulmonary abscess formation.

Intravenous injections of the soluble sodium salt of sulfathiazole or sulfapyridine seemed to benefit calves that showed severe pulmonary distress and general bodily weakness. This therapy possessed the obvious advantage of establishing effective blood concentrations immediately and avoided the time required for absorption of sulfonamides from the gastrointestinal tract. Intravenous therapy was always accompanied by oral administration of the first of the divided doses of drug so that absorption from the gastrointestinal tract would soon replace the drug being excreted from the blood stream.

SUMMARY

1) Ninety-five clinical cases of calf pneumonia treated during 1942 to 1944 were divided by rotation into four groups to receive sulfathiazole, sulfapyridine, sulfadiazine, and no treatment. The following mortality was noted in each group: sulfathiazole, 16 per cent; sulfadiazine, 20.8 per cent; sulfapyridine, 25 per cent; untreated, 68.2 per cent. Without doubt, the sulfonamide therapy decreased the percentage of mortality as compared to the control group, but the differences in percentage recoveries between the three drug groups were too small to be statistically significant.

2) The treatment with sulfathiazole resulted in a shorter duration of therapy, *i.e.*, a quicker recovery, than was obtained with sulfapyridine and sulfadiazine.

3) The average concentrations of free sulfonamides present in the blood streams of clinical cases during treatment were: sulfathiazole, 2.88 mg. per 100 cc. of whole blood; sulfapyridine, 6.63 mg. per 100 cc.; sulfadiazine, 6.38 mg. per 100 cc.

4) Fewer relapses occurred among the calves treated with sulfathiazole than with sulfapyridine and sulfadiazine. The number of cases was too small to render the above differences statistically significant; however, an important trend may be indicated by the above data.

5) In general, the hematologic values in the treated clinical cases did not show clinically significant variations attributable to the sulfonamide compounds during the brief period that these drugs were administered.

6) Values for the urinary and fecal concentrations of excreted sulfonamides were determined on 26 patients during therapy.

7) The average percentages of acetylated sulfonamides present in the blood of 26 recovered clinical cases was approximately 21 per cent for all three compounds. The acetylated sulfonamide present in the urine varied as follows: sulfathiazole, 37 per cent; sulfapyridine, 49 per cent; sulfadiazine, 67 per cent. In the feces, the acetylated drugs were: sulfathiazole, 24 per cent; sulfapyridine, 41 per cent; sulfadiazine, 20 per cent.

8) The blood urea nitrogen determinations served only as a test for confirming clinical observations of drug toxicity.

9) The chief toxic condition in calves attributable to sulfonamide therapy was a stoppage of urinary excretion due to the precipitation of sulfonamides in the urinary system. Clinical signs of toxicity were: inappetance, depression, frequent urination with straining, hematuria, restlessness, oliguria leading to anuria with sulfathiazole hyperesthesia, and peripheral neuritis.

10) Bacteriologic cultures were made of the tissues from 29 out of 30 calves that died during the experiment, 14 treated and 15 untreated. The bacterial organisms isolated were: *Pasteurella multocida*, 6 cases;

Corynebacterium pyogenes, 4 cases; *Staphylococcus aureus*, 4 cases; *Pseudomonas aeruginosa*, 4 cases; streptococci, 2 cases; *Escherichia coli*, 2 cases; and 7 cases, including two deaths due to renal concretions, were sterile. Representatives of each of the above six species of bacteria were isolated from both the treated and the untreated fatalities.

11) Five of 9 newborn, normal calves responded to intranasal and intratracheal inoculations of infective tissue in the manner described by Baker in his experiments with a virus of calf pneumonia.

12) A maximum dosing schedule for the administration of sulfonamides in divided doses to calves up to 4 months of age would seems to be: sulfathiazole, 0.214 Gm./kg. (1.5 gr./lb.) for two to three days followed by 0.143 Gm./kg. (1.0 gr./lb.) for two to three days; sulfapyridine, 0.143 Gm./kg. for one to two days followed by 0.066 Gm./kg. (0.66 gr./lb.) for two to three days; and sulfadiazine, 0.143 Gm./kg. for two to three days followed by 0.066 Gm./kg. for two to five days. Briefer periods of therapy should be employed whenever the clinical response of the patient permits.

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The Genesis of Bovine Udder Infection and Mastitis
II. The Occurrence of Streptococcal Infection in a Cow Population During a Seven-Year Period and Its Relationship to Age

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BOVINE UDDER INFECTION due to *Streptococcus agalactiae* is known to be widely prevalent, of nonuniform incidence, and usually to cause an inflammation of a chronic or subacute nature. Such infection is commonly considered to spread slowly through a cow population. Specific studies[9] have indicated that transmission takes place slowly and that not every animal becomes infected with equal facility. Hucker and Harrison[6] observed the number of cows free of streptococcal infection entering three herds and the percentage of these that developed infection in a period of about three years. The spread of infection was not rapid, and they considered that the results indicated that the lower the percentage of cows discharging mastitis streptococci, the greater was the possibility of infection being on the decrease in the herd, and the greater the possibility of clean cows remaining free of infection upon being added to the herd.

On the other hand, Stableforth[21] pointed out that one reason why streptococcal mastitis is of outstanding importance is that it is contagious and, for reasons not yet established, may suddenly assume a severe form affecting a large proportion of a herd. However, the same author[23] noted later that, when a clinical outbreak is observed, it is not generally known whether there has been a recent spread, or whether a number of previously infected cows have simultaneously shown clinical manifestations through environmental causes. To our knowledge, the only sudden outbreak of udder infection due to *Str. agalactiae* satisfactorily observed was reported by Hastings, Beach, and Johnson[5]. These investigators found that, in a herd of 24 cows maintained free of streptococcal infection of the udder until about the middle of the third lactation period for most of the animals, an outbreak of *Str. agalactiae* infection occurred that affected 13 (54.2%) of the cows in a period of seven months.

Largely because of the naturally occurring, increasing-with-age pattern shown by natural streptococcal infection and the irregularities attending attempts to establish such infection by artificial means, it is usually considered that some predisposing condition is necessary for the natural establishment of streptococcal infection. We[16] have reviewed several theoretically predisposing conditions in the light of data showing that while streptococcal infection increased with age, staphylococcal infection did not. That analysis showed that teat patency and obvious injury to the teat could play only minor rôles in the genesis of udder infection. It was further indicated that a closer scrutiny of the relationship of age to udder infection might reveal significant information.

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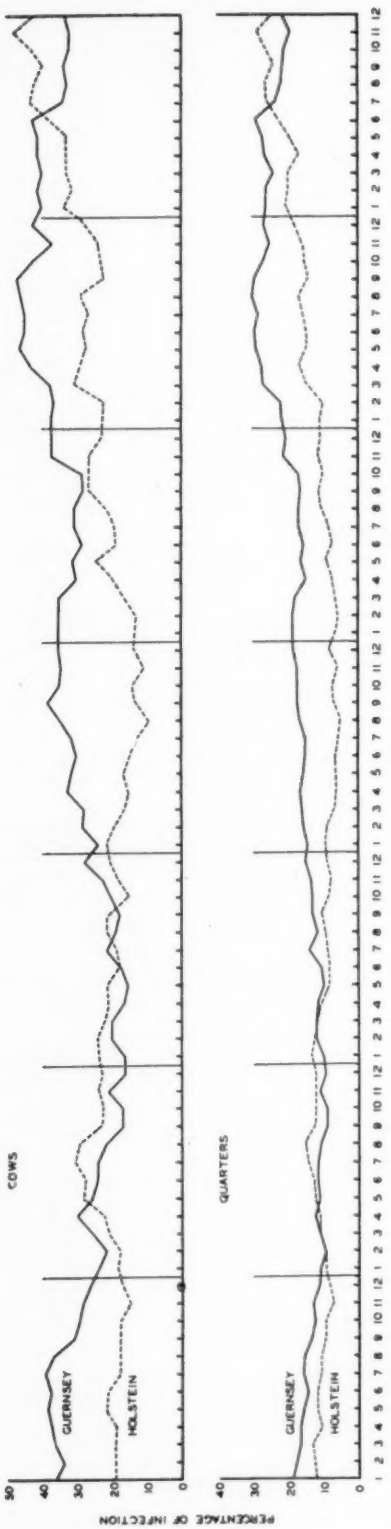

Fig. 1—The monthly incidence of streptococcal infection in the Guernsey and Holstein-Friesian herds as the percentage of cows and as the percentage of quarters.

The present analysis was undertaken for the dual purpose of determining what actually takes place in a herd of cows, some of which were naturally infected with *Str. agalactiae*, over a period and to investigate further the relationship of age to the occurrence of udder infection.

METHODS

This is the second of several reports dealing with the over-all picture of udder infection and mastitis for a seven-year period (1938 to 1944 inclusive) in the herd of the Dairy Research Station. This unit actually consisted of two herds: one of approximately 60 Guernsey milking animals and the other of approximately 60 Holstein-Friesian milking animals. All but a few of the animals in each herd were purebred.

The herds were housed separately in modern, one-story barns, of identical size and construction, parallel to one another and about 20 ft. apart. The floors were concrete, the walls hollow tile faced with cement, the ceiling 9 ft. from the floor, and the roof peaked and covered with asbestos shingles. Thermostatically controlled, forced ventilation was provided in each barn. The stalls were of the Hoard Comfort type and arranged in two rows facing inward to a central feeding alley. Each stall measured approximately 3.5 by 6 ft. and was separated from the adjoining stalls by a concrete curb 12 in. high and 4 in. wide extending from the manger to the gutter. Ample quantities of bedding, almost exclusively sawdust and shavings from green wood, were used most of the time.

With very few exceptions, both herds were self-contained for the duration of this study and for at least the three preceding years. Replacements were, therefore, mainly offspring of the herds and raised on the premises. Both herds were under the same management, were milked by the same men (at any one time) twice a day (at 4 a.m. and 3 p.m.). One lb. of good guality, 18 per cent dairy ration was fed for each 3 lb. of milk produced by the Guernseys, and 1 lb. of the same feed for each 4 lb. of milk produced by the Holstein-Friesians. Roughage (primarily grass silage supplemented by corn silage, hay, and pasture) was fed *ad libitum*. Each cow was trained to its own stall and changes kept at a minimum. The herds were pastured separately but passed through the same alleyway and barnyard.

The general sanitation was good but of a practical nature. During the period of hand milking (January, 1938, to May, 1942), each man was assigned a string of approximately 10 cows in each herd. Each milker washed the udders of his own cows, using one bucket of hot chlorine solution (200 p.p.m.) and one cloth for each string of 10 cows. Each cow was washed separately just prior to being milked, so that the milker's hands were at least rinsed before he milked each cow. During the period of machine milking (June, 1942, to December, 1944), one man operated the machines and the other men performed the tasks of washing udders, foremilking, stripping, etc. Each udder was washed with a separate clean cloth and clean, hot chlorine solution, but several cows were washed in advance of the machine in order to prepare the udders for milking, The teat cups of the milking machine were rinsed in cold water and then in hot chlorine solution prior to being used on each cow. Machines were thoroughly rinsed between herds and were dismantled and thoroughly cleaned after each milking period. Throughout this study, the Guernsey herd was milked first and the Holstein-Friesian herd second.

Except in cases of severe acute mastitis, no treatment for mastitis or udder infection was applied to any cow during the first six years and seven months of the seven-year study period. The previously reported[15] investigation of the value of penicilin for this purpose was made in the latter part of the seventh year (1944) and during 1945. This treatment study involved only the Holstein-Friesian herd.

From the onset of the study, a fully equipped laboratory and living quarters for the staff were maintained on the premises. Beginning in November, 1937, strict foremilk from each quarter of each cow in the herd was examined bacteriologically at thirty- to sixty-day intervals. No examinations were made during the dry period. Quarter samples of colostrum were obtained prior to freshening and were examined bacteriologically. The regular thirty- to sixty-day examinations were begun seven to ten days after freshening. Samples were plated in a 1 : 20 dilution in meat-infusion, blood agar. Bacteria were identified as to genus after incubation of the plates at 37 C. for forty-eight hours. At intervals, the streptococci were isolated and identified as to species, as previously reported[14].

During this study, 25,248 samples of strict foremilk (approximately 20 cc.) were drawn and examined by the author. The present report deals only with streptococcal infection: a quarter was considered to be infected with streptococci if one or more colonies of streptococci were present in the blood-agar culture regardless of the presence of other bacteria. A cow was considered to be infected with streptococci if one or more quarters were infected in the above sense. In relating the month-to-month incidence of infection, it was found necessary to consider the streptococci as a unit, rather than to consider each species independently. Streptococci of each of Minett's groups I, II, and

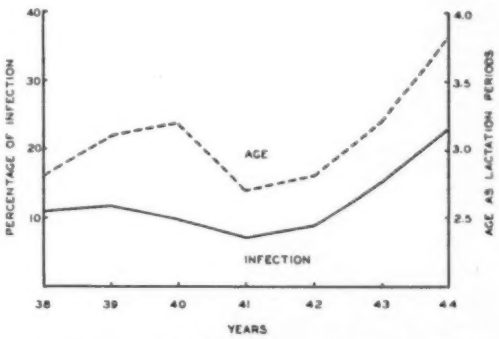

Fig. 2—Guernsey herd—average incidence of streptococcal infection of quarters and average age by years.

III[13], and some atypical forms were identified during the study, but at any one time at least 90 per cent (and usually 95%) of the total streptococcal infection in the herd was due to members of Minett's group I, *Str. agalactiae.* Inasmuch as streptococci other than *Str. agalactiae* constituted an insignificant portion of the total streptococcal infection at any time, the unqualified use of the terms "streptococci" and "streptococcal" in this report can be regarded as referring to *Str. agalactiae.* Because animals were at times examined at sixty-day intervals, and because no examinations were made during the dry period, in calculating the monthly incidence of infection, the infection present at the last previous examination was assumed to be present during either of these intervals.

RESULTS

At the beginning of the period of observation, in January, 1938, 36.7 per cent of the Guernsey cows and 19.4 per cent of the Guernsey quarters were infected with streptococci, and 19.6 per cent of the Holstein-Friesian cows and 12.6 per cent of

the Holstein-Friesian quarters. Figure 1 shows the monthly incidence of streptococcal infection in both herds as the percentage of cows and as the percentage of quarters infected. In the Guernsey herd, the percentage of infected quarters decreased during 1938 to about 11 per cent, remained at about 11 per cent during 1939, gradually increased during 1940 to about 15 per cent, continued to increase in 1941 to about 19 per cent, decreased during the middle part of 1942 to about 16 per cent, and then increased to about 30 per cent by the middle of 1943. From the middle of 1943 to the end of 1944, there was a gradual decline in the percentage of quarters infected with streptococci. In the Holstein-Friesian herd, the incidence of infection decreased during 1938 to about 9 per cent, increased during 1939 to about 12 per cent, decreased in 1940 to about 9 per cent, and decreased during the first half of 1941 to about 5 per cent. In the latter part of 1941 and the early part of 1942, the incidence of infection increased slightly, and from then on until the end of 1944, there was an almost constant increase to about 30 per cent. These trends are shown in table 1 as the average incidence for each of the seven years. It can be seen quite clearly that a difference in the incidence of infection by years existed in the two herds.

At the beginning of the period of observation, there were 60 Guernsey cows and 56 Holstein-Friesian cows in the milking line. The two herds were maintained at about 60 milking animals each, and the average monthly number of cows in each herd for each of the seven years is shown in table 1. In all, 322 milking animals were studied. Two hundred and six cows were added to the herds during the seven years; 198 of these were just beginning their first lactation period (table 2), and 8 were added after their first lactation period had begun; most of these were in their second or subsequent lactation periods (table 3). Two hundred and twenty-four animals were removed from the herds during the seven-year period (table 5) and, of these, the Guernsey cows averaged 4.0 lactation periods completed and the Holstein-Friesian cows 2.9 lactation periods completed.

The incidence of infection in animals added to each herd is shown in tables 2 and 3. In all, 10.2 per cent of the quarters of cows added to the Guernsey herd and 8.0 per cent of the quarters of cows added to the Holstein-Friesian herd were infected with streptococci.

TABLE 1—The Average Number, Age, and Incidence of Streptococcal Infection of Cows in the Guernsey and Holstein-Friesian Herds Based on Monthly Observations

	Guernsey						Holstein-Friesian					
			Incidence of infection						Incidence of infection			
			Cows		Quarters				Cows		Quarters	
Years	No.	Age*	No.	(%)	No.	(%)	No.	Age*	No.	(%)	No.	(%)
1938	64	3.1	22	(34.4)	39	(15.3)	55	2.8	10	(18.8)	24	(10.9)
1939	64	3.0	14	(22.5)	27	(10.7)	54	3.1	13	(24.1)	26	(12.2)
1940	59	3.2	12	(19.6)	29	(12.1)	55	3.2	11	(20.6)	22	(10.0)
1941	60	3.3	19	(31.8)	39	(16.3)	60	2.7	9	(14.8)	17	(7.0)
1942	58	3.4	19	(32.2)	40	(17.6)	58	2.8	12	(20.6)	20	(8.8)
1943	50	3.7	21	(42.1)	53	(26.9)	55	3.2	15	(26.2)	33	(14.8)
1944	48	3.6	18	(36.8)	46	(24.0)	57	3.8	22	(38.2)	52	(22.9)

*As lactation periods.

Of the cows added at the beginning of their first lactation period, 22.8 per cent of Guernsey cows, 9.2 per cent of Guernsey quarters, 10.4 per cent of Holstein-Friesian cows, and 4.2 per cent of Holstein-Friesian quarters were infected with streptococci (table 2). Of the 52 quarters (both breeds) found to be infected with streptococci at

the beginning of the first lactation period, the infecting organisms in 21 were identified: only one of the 21 identified organisms proved to be *Str. agalactiae*. Of these 52 streptococcal infections, 38 disappeared within the first two months of lactation (10 were *Streptococcus dysgalactiae*, 4 were *Streptococcus uberis*, and 24 were unidentified), six disappeared in the third or fourth month of lactation (3 were *Str. uberis* and 3 were unidentified), two disappeared in the fifth or sixth month of lactation (both *Str. uberis*), three persisted for nine or more months (1 *Str. agalactiae* and 2 unidentified), and for three (1 *Str. uberis* and 2 unidentified), the duration was not known because the animals were disposed of soon after freshening and while the infection was still present (table 4).

Of the animals added to the herds after their first lactation period had begun or in their second or subsequent lactation period (table 3), the 1 Guernsey cow was infected in four quarters with *Str. agalactiae*, and 6 of 7 Holstein-Friesian cows were infected in 18 quarters, of which the infecting organisms in 13 were known to be *Str. agalactiae*.

TABLE 2—The Number of Cows Added to the Guernsey and Holstein-Friesian Herds at the Beginning of Their First Lactation Period, Their Status with Regard to Streptococcal Infection, and the Identity of the Streptococci

Herd	Total cows added	Streptococcus-infected					
				Streptococcal identity*			
		Cows	Quarters	Group I	Group II	Group III	Unidentified
Guernsey	92	21 (22.8%)	34 (9.2%)	1	3	7	23
Holstein-Friesian	106	11 (10.4%)	18 (4.2%)	0	7	3	8
Total	198	32 (16.2%)	52 (6.6%)	1	10	10	31

*According to Minett[12]: Group I, *Str. agalactiae*; Group II, *Str. dysgalactiae*; and Group III, *Str. uberis*.

Of 224 cows removed from the herds (table 5), 45.4 per cent of the Guernsey cows, 24.5 per cent of the Guernsey quarters, 33.3 per cent of Holstein-Friesian cows, and 17.3 per cent of Holstein-Friesian quarters were infected with streptococci. Of the 187 infected quarters, the infecting organism was identified in 145, of which 127 (87.6%) were *Str. agalactiae*.

DISCUSSION

So far as can be judged from the literature, this is the first time that a large group of cows, some of which were naturally infected with *Str. agalactiae*, have

TABLE 3—The Number of Cows Added to the Guernsey and Holstein-Friesian Herds After Their First Lactation Had Begun, Their Status with Regard to Streptococcal Infection, and the Identity of the Streptococci

Herd	Total cows added	Streptococcus-infected					
				Streptococcal identity*			
		Cows	Quarters	Group I	Group II	Group III	Unidentified
Guernsey	1	1 (100.0%)	4 (100.0%)	4	0	0	0
Holstein-Friesian	7	6 (85.7%)	18 (64.3%)	13	0	1	4
Total	8	7 (87.5%)	22 (68.7%)	17	0	1	4

*According to Minett[12]: Group I, *Str. agalactiae*; Group II, *Str. dysgalactiae*; and Group III, *Str. uberis*.

been maintained and examined bacteriologically at frequent intervals for a significant period with the intention of studying the natural spread of infection. Two herds of equal size but of different breeds have been maintained separately under almost identical circumstances on the same farm

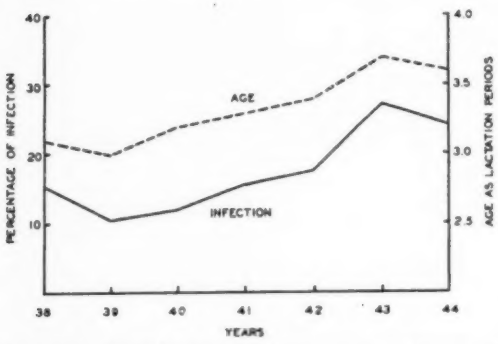

Fig. 3—Holstein-Friesian herd—average incidence of streptococcal infection of quarters and average age by years.

for a period of seven years. The natural incidence of streptococcal infection (90% or more of which was due to *Str. agalactiae*) at the onset of the study was comparable to that observed in certain herds by other investigators[1, 2, 8, 20, 21]. If it were possible to classify the two herds according to the degree of infection at the onset of the study, the Guernsey herd with 36.7 per cent of the cows infected would probably be classed as "high", and the Holstein-Friesian herd with 19.6 per cent of the cows infected would probably be classed as "moderate".

Each year, enough first calf heifers were added to each herd to offset the number of animals removed; in only a few cases were animals other than heifers beginning their first lactation period added to the herd. Although the incidence of streptococcal infection of quarters in the first calf heifers at the time of freshening was nearly 7 per cent for the period of seven years, most of these infections were not due to *Str. agalactiae*, and most of them did not persist for more than a few months. This observation is also similar to that recorded by other investigators[1, 8, 10, 11, 17, 19].

Animals were removed from the herds for various reasons, of which mastitis was one only to the extent to which it is recognized in ordinary herd management. Information obtained on udder infection and mastitis in this study was not available to the individuals responsible for the management of the herds. In both herds, the incidence of infection in animals removed was generally no higher than the incidence of infection within the herds, and 87.6 per cent of the identified streptococcal infections removed from the herds were due to *Str. agalactiae*. Thus, it can be seen that the source of replacements was very low in persistent streptococcal infection and in *Str. agalactiae* infection, and that after animals had been in the herd for a length of time, they became infected somewhat in relation to the incidence of infection in the herds.

The monthly incidence of infection (fig. 1) behaved differently in the two herds:

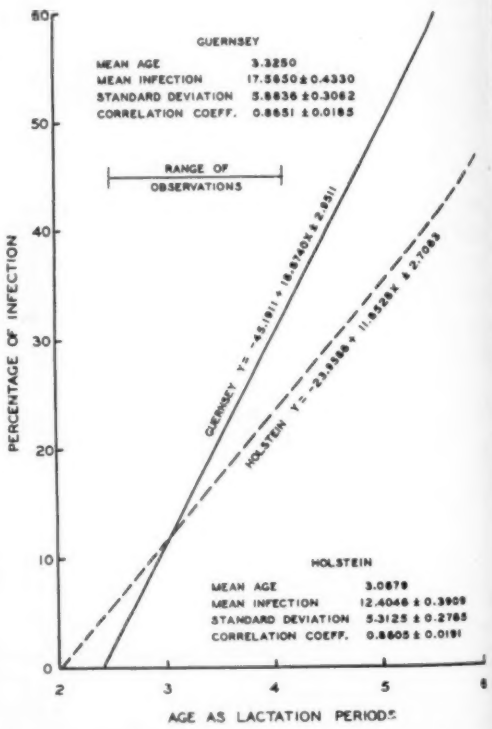

Fig. 4—Graph of prediction equations for streptococcal infection of quarters for each herd based on 84 monthly observations.

at times the Guernsey herd had a greater incidence of infection, at times the herds were about equal, and at times the Holstein-Friesian herd had a greater incidence of infection. The lowest incidence of infection of quarters in the Guernsey herd was in October, 1939, from which it rose gradually to the highest incidence in August, 1943: or from 9 per cent to 30 per cent in forty-seven months. The lowest incidence of infection of quarters in the Holstein-Friesian herd was in August, 1941, from which it rose gradually to the highest incidence in November, 1944; or from 5 per cent to 29 per cent in thirty-nine months. Thus, in these two herds, during the seven years of observation, the spread of streptococcal infection was very slow.

There were times, of course, when the incidence of streptococcal infection doubled within a period of twelve months. This was more apparent as the percentage of infected cows increased: in eight months from November, 1938, to July, 1939, the number of Holstein-Friesian cows infected rose from 15 per cent to 30 per cent; in twelve months from September, 1940, to September, 1941, the number of Guernsey cows infected rose from 18 per cent to 36 per cent; and in nine months from August, 1941 to May, 1942, the rate of infection in Holstein-Friesian cows rose from 10 to 24 per cent. Similar increases in the percentage of quarters infected with streptococci occurred. Nevertheless, at no time was the spread of infection comparable to the instance observed by Hastings, Beach, and Johnson[5], in which the incidence rose from 4.2 per cent to over 50 per cent of the cows in a period of seven months.

In addition, it was observed that increases and decreases in the incidence of infection (considered either as quarters or as cows) were not closely related: the low points in the percentage of quarters infected in each herd were separated by twenty-one months and the high points were separated by fourteen months. Thus, such general management practices as stabling, feeding, and milking, which were much the same in each herd, would not be expected to have contributed significantly to the spread of infection (this will be discussed in detail in a subsequent publication).

It is also to be observed that the incidence of infection at times remained more or less constant for months, and then either increased or decreased. One outstanding illustration was in the percentage of Holstein-Friesian cows infected from January, 1938, to July, 1939: during the first fourteen months of this time, the infection remained fairly constant (at about 20%) and then, in the next five months, increased to 30 per cent. The converse is also to be observed at other times. It is not easily understood, therefore, how the increase and decrease of infection could bear any close relationship to the incidence observed at any one time as suggested by Hucker and Harrison[6]. On the contrary, it would appear more reasonable, if new infection depended mainly on the amount of existing infection, for the incidence of infection always to be increasing, and to increase ever more rapidly as the over-all incidence became greater.

On the other hand, the spread of streptococcal infection within a herd should be related to those factors or forces of major concern in the genesis of infection of the mammary gland. As a result of the ob-

TABLE 4—The Identity and Duration of Streptococcal Infection in Cows Added to the Herd at the Beginning of Their First Lactation Period

Streptococcal identity*	Duration of infection (months)						
	1-2	3-4	5-6	7-8	9 or more	Unknown	Total
Group I	—	—	—	—	1	—	1
Group II	10	—	—	—	—	—	10
Group III ..	4	3	2	—	—	1	10
Unidentified .	24	3	—	—	2	2	31
Total ..	38	6	2	—	3	3	52

*According to Minett[12]: Group I, *Str. agalactiae*; Group II, *Str. dysgalactiae*; and Group III, *Str. uberis*.

servation that streptococcal infection increases with age, and as a result of the difficulties or inconsistencies attending attempts to infect the mammary gland artificially, it has been suggested that some predisposing factor or factors exist that are necessary to the natural establishment of streptococcal infection of the udder. We[16] have recently reviewed the prevailing theories concerning predisposing factors and their relationship to the genesis of udder infection and reached the conclusion that a closer scrutiny of the age relationship might be of value.

Figures 2 and 3 show the average yearly incidence of streptococcal infection by quarters, and the average yearly age (as lactation periods) of the two herds. It is obvious that the actual incidence of infection is not the same in the two herds each year, but that the relationship of the incidence of infection to the average age is very close in both herds during the seven-year period. This relationship, when subjected to statistical analysis on the basis of 84 monthly observations, is highly significant (fig. 4). From this, it can be seen that the percentage of quarters infected with streptococci increased and decreased directly in relation to the average age of the herd. The correlation is so close that little room is left for other influences to be of significance. This same relationship is to be seen when streptococcal infection is considered on a cow basis, rather than on a quarter basis (fig. 5), so that the rise and fall of the percentage of cows infected was also in direct relation to the rise and fall of the average age of the herd. Age, then, or some function of age, was the major limiting factor in the spread of streptococcal infection in these herds during the seven-year period of observation.

Another indication of the regularity with which infection occurred, or the pattern that infection laid down, is to be seen in the relationship of the incidence of quarter infection to the incidence of cow infection. Statistically, on an 84-month basis, this relationship is highly significant in that the following correlation data were obtained: Guernsey herd, correlation coefficient 0.8835 ± 0.0161 (standard deviation 8.1759 ± 0.4255) and Holstein-Friesian herd, correlation coefficient 0.9479 ± 0.0075 (standard deviation 7.7223 ± 0.4019). The average incidence of infection for the eighty-four months was 17.5650 per cent of quarters and 31.3462 per cent of cows infected with streptococci in the Guernsey herd, and 12.4046 per cent of quarters and 23.3686 per cent of cows infected with streptococci in the Holstein-Friesian herd. This means that a ratio of 1.7846 quarters infected for each cow infected in the Guernsey herd, and a ratio of 1.8839 quarters infected for each cow infected in the Holstein-Friesian herd, were maintained uniformly over the 84-month period. No similar observation is on record, but it is of interest that Stableforth[2]

TABLE 5—The Total Number and Average Age of Cows Removed from the Guernsey and Holstein-Friesian Herds, Their Status with Regard to Streptococcal Infection, and the Identity of the Streptococci

Herd	Total cows removed	Av. age*	Streptococcus-infected					
					Streptococcal identity†			
			Cows	Quarters	Group I	Group II	Group III	Unidentified
Guernsey	110	4.0	50 (45.4%)	108 (24.5%)	89	1	6	12
Holstein-Friesian	114	2.9	38 (33.3%)	79 (17.3%)	38	0	11	30
Total	224	3.5	88 (39.3%)	187 (20.9%)	127 (87.6%)	1	17	42

*As lactation periods

†According to Minett[12]: Group I, *Str. agalactiae;* Group II, *Str. dysgalactiae;* and Group III, *Str. uberis.*

noted that infected animals average two infected quarters, and Munch-Petersen[11] presented data obtained by several other investigators (Engler, Simons, Steck, Seelemann, Stylianopoulos) from which we have calculated that the following ratios of infected cows to infected quarters were observed: 1 : 1.6, 1 : 1.6, 1 : 2.1, 1 : 2.4, and 1 : 1.9.

In order to bring out the marked influence of age, as well as to indicate the presence of other influences of possible significance, the age factor, as it affects the data in figure 1, has been nullified by applying the respective prediction equations to each monthly observation of streptococcal infection and age. Thus, in figure 6, the actual incidence of streptococcal infection for any month is shown as the amount that it was above or below the predicted incidence for that month according to the average age of the herd at that particular time. It is then seen that the monthly incidence of streptococcal infection remained uniform for most of the seven-year period within a narrow band (approximate standard error of estimate) of 10 per cent on the basis of cows infected, and 6 per cent on the basis of quarters infected. The generally uniform incidence of infection* is broken by a possibly significant high incidence of infection in Guernsey cows in 1938, by a probably significant low incidence in both cows and quarters of each breed in 1940, and by a possibly significant high incidence in both cows and quarters of each breed in 1943. The possible significance and meaning of these exceptions to the uniformity of the incidence of infection will be dealt with in subsequent publications.

It is of equal or greater importance, however, to know how this age relationship affected individual cows within the herds and, for this purpose, we have selected the records of all those animals which had freshened, for the first time, during the first year of the study and which had survived, and been under constant study for at least six years. There were found to be 17 cows (8 Guernsey and 9 Holstein-Friesian) that met this specification. Eleven (64.7%) became infected with streptococci at some time during the six-year period, and 6 (35.3%) showed no streptococcal infection during the six-year

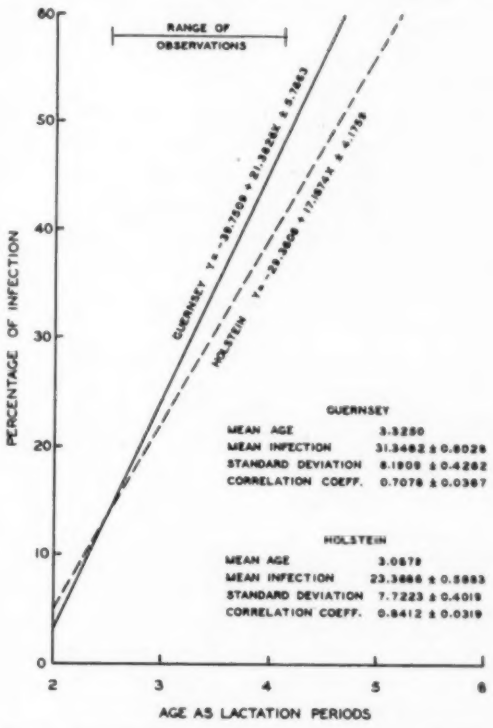

Fig. 5—Graph of prediction equations for streptococcal infection of cows for each herd based on 84 monthly observations.

period. In figure 7 are shown the occurrence of infection in the 11 cows that became infected and the average daily milk production† of these and of the 6 cows that did not become infected during the six years. All the records have been given a common beginning so that we can deal in terms of years of milking life instead of in calendar years.

* The difference in uniformity of cow infection and quarter infection is due largely to the fact that, in dealing with quarters, each quarter is considered separately as infected or uninfected, but in dealing with cows, a cow is considered to be infected whether one or more quarters are involved.

† As pounds of 4 per cent fat-corrected milk.

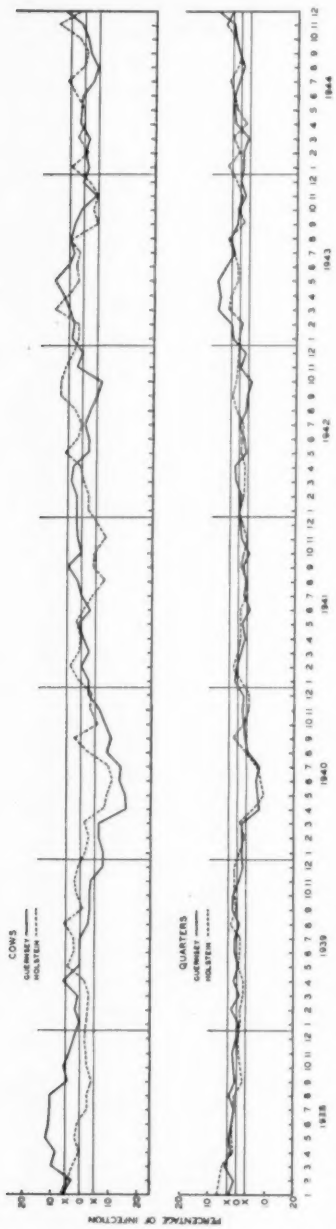

Fig. 6—The monthly incidence of streptococcal infection after nullification of the age factor by applying the prediction equations to each monthly observation. Actual infection is shown as above or below the predicted values (0) and in relation to the approximate standard errors of estimate (X).

It is to be seen that, with two minor exceptions (which are not considered hereafter), the infection history of the group that eventually became infected began on the first month of the third year of milking life. From then to the end of the sixth year of milking life, the incidence of infection by quarters regularly increased to 66 per cent. The regularity of the increase in infection is clearly shown by the fact that the incidence stayed within a straight narrow band of 16 per cent at all times. In terms of the year of milking life during which infection first appeared, it was found that 2 cows became infected in the third year, 4 in the fourth year, 2 in the fifth year, and 3 in the sixth year. In terms of the lactation period during which infection first appeared, it was found that 2 became infected in the second period, 1 in the third period, 5 in the fourth period, 2 in the fifth period, and 1 in the sixth period. As to the portion of the lactation period in which infection was first observed, it was found that eight of the infections appeared in the first third of lactation, one in the middle third, and two in the last third.

Thus, with individuals, there was a period at the beginning of the milking life that was free, or relatively free, of streptococcal infection. This period was at least two years for all of the cows, and at least two lactation periods for 9 of the 11 cows. (One-third of the cows did not become infected at all in the six-year period.) The observation that the incidence of streptococcal infection of quarters would be zero if the average age of the herd was two lactation periods (fig. 4) is thereby confirmed and partly explained. With individuals, as with the herd, this free period was followed by a period during which infection appeared and regularly increased with the passing of time. There can be no reasonable doubt, therefore, that age, or some function of age, was the major limiting or predisposing factor in the genesis of streptococcal infection in the 322 cows in two herds that were observed during a seven-year period. Furthermore, there can be no reasonable doubt that the age factor is a function of age, in the sense of some change taking place within the body with the passing of time, rather than being an effect of age *per se*, in the sense of a mere passing of time creating

a greater opportunity for exposure to infection.

Of the six theories advanced by way of explanation of the increasing-with-age incidence of natural streptococcal infection and the probably related difficulties attending attempts to infect the udder artificially, teat patency and obvious injury have been relieved of further major consideration by evidence previously presented[16]. The data presented in the present report, showing that the relationship between age and infection is a straight-line relationship beginning more than one year after the onset of milking life, rather than a curved-line relationship beginning at, or near, the onset of milking life and increasing in intensity with the passing of time, make the degree-of-exposure theory untenable.

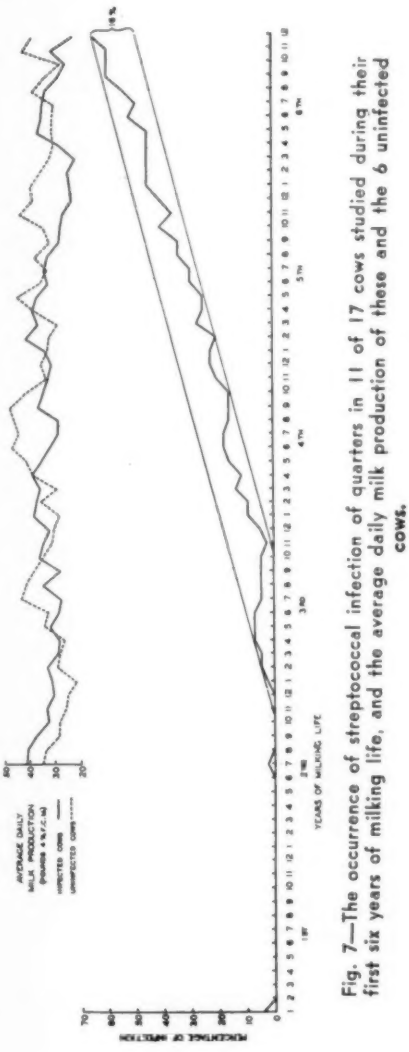

Fig. 7—The occurrence of streptococcal infection of quarters in 11 of 17 cows studied during their first six years of milking life, and the average daily milk production of these and the 6 uninfected cows.

Nonspecific mastitis was suggested by Peterson and Hastings[13] as a primary or predisposing condition following which *Str. agalactiae* causes chronic mastitis as a secondary invader, principally because of the fact that quarters so affected were unusually easy to infect artificially. However, a subsequent report by Hastings, Beach, and Johnson[5], concerning an outbreak of *Str. agalactiae* infection in a herd of 24 cows some of which were affected with nonspecific mastitis, showed clearly that the prior occurrence of this condition was not necessary to natural infection of the bovine mammary gland with *Str. agalactiae.*

The prior-sensitization theory of Jones and Little[7], based mainly on the observation that when the number of streptococci artificially introduced into the normal bovine mammary gland is sufficiently small, infection usually takes place only after repeated introductions of the organisms, essentially contends that some change occurs within the animal body as a result of exposure to streptococci which allows the establishment of streptococci within the body. In so far as this theory is part of the degree-of-exposure theory, it is untenable for the same reasons.

The hormone theory of Francis[4] introduced the thought that some change might occur within the bovine mammary gland with the passing of time, as an effect of the part played by hormones in the growth and function of the gland, that would make it more susceptible to infection with streptococci. In postulating this theory, Francis drew heavily on the nonspecific mastitis observations of Peterson and Hastings[13], although recognition was made of the fact that that condition had not been defined beyond being a state of chemical abnormality (primarily chlorides) of the secretion of a gland in the absence of strep-

tococcal infection. As has already been mentioned, the later observations of the authors of nonspecific mastitis have shown that it does not necessarily predispose to subsequent streptococcal infection, and this clearly disturbs the hormone theory.

Nevertheless, the primary purpose of Francis was to stress the matter of host susceptibility, and this was merely hypothetically expressed in terms of hormonal action. On the basis of host susceptibility, the hormone theory and the observations of the present study meet, in that the one suggests, and the other demonstrates, that streptococcal infection is limited largely by the receptiveness of the host, and that this was due to some change taking place within the body not dependent on exposure to environment (streptococci). Therefore, although there are no observations available at present on which to connect the action of hormones directly to the occurrence of streptococcal infection, the hormone theory of Francis stands as the only valid one of the six predisposing factor theories considered.

Other theories could, of course, be built upon the observation that a function of age was the major predisposing or limiting factor in the herds studied, for hormonal action is by no means the only possibility. Yet no clear purpose would be served, inasmuch as the results already show that the age factor is an expression of host susceptibility, that this susceptibility appeared in the individuals (of the population studied) at different times so that the susceptibility of the population (as a whole) increased and decreased in direct relation to the average age, and that the site of action is the infection phase of the invasion-infection-inflammation concept of the genesis of bovine mastitis[16].

SUMMARY

Two herds of equal size (approximately 60 cows each), but of different breeds (Guernsey; Holstein-Friesian), have been maintained separately under almost identical circumstances on the same farm for a period of seven years for the purpose of studying the natural spread of streptococcal infection. Each year enough first-calf heifers were added to each herd to offset largely the number of animals removed; in only a few cases were animals other than heifers beginning their first lactation period added to the herd. Animals were removed from the herds for various reasons, of which mastitis was one only to the extent to which it is recognized in ordinary herd management.

The natural incidence of streptococcal infection (90% or more of which was due to *Streptococcus agalactiae*) at the onset of the study was 36.7 per cent of cows in the Guernsey herd and 19.6 per cent of cows in the Holstein-Friesian herd. The spread of infection within the herds was very slow and did not appear to be related to general management practices such as stabling, feeding, and milking, because increases and decreases in the incidence of infection in the two herds did not coincide.

It was observed, however, that the rise and fall of infection were related to the average age of the herd. Statistically, this relationship was highly significant (correlation ratios: Guernsey quarters, 0.8651 ± 0.0185; Guernsey cows, 0.7078 ± 0.0367; Holstein-Friesian quarters, 0.8605 ± 0.0191; Holstein-Friesian cows, 0.8412 ± 0.0319) and indicated that age, or some function of age, was the major predisposing or limiting factor in the spread of streptococcal infection in the herds during the seven-year period of observation.

Examination of the records of 17 individuals that had freshened for the first time during the first year of the study, and that had been observed for the first six years of their milking life, revealed that approximately two-thirds of the animals became infected with streptococci. Streptococcal infection began after the second year of milking life and increased regularly (within a straight band of 16%) to 66 per cent of quarters at the end of the sixth year of milking life.

Inasmuch as the age relationship was a straight-line relationship beginning more than one year after the onset of milking life, rather than a curved-line relationship beginning at or near to the onset of milking life and increasing in intensity with the passing of time, the age factor must be a function of age (in the sense of some change taking place within the body with the passing of time) rather than being an effect of age *per se* (in the sense of a mere passing of time creating a greater opportunity for exposure to the infecting microörganisms).

Six predisposing factor theories advanced by various investigators in explanation of the increasing-with-age incidence of natural streptococcal infection, and the irregularities attending attempts to establish such infection by artifical means, were discussed. Two of these theories, teat patency and obvious injury to the teat, have been relieved of further major consideration by information previously presented; two theories, degree-of-exposure and prior-sensitization (in so far as it is related to degree-of-exposure), are rendered untenable by the observation that the age factor is a function of age; and one theory, nonspecific mastitis, is noted to have been refuted by the later observations of its authors.

The remaining theory, the hormone theory of Francis, which introduced the thought that some change might occur within the mammary gland with the passing of time, as an effect of the part played by hormones in the growth and function of the gland, that would make it more susceptible to streptococcal infection, now stands as the only predisposing factor theory that can be considered valid.

DISCUSSION‡

DR. ERWIN JUNGHERR (Storrs, Conn.).—Were they *Streptococcus agalactiae* or straight Streptococcus?

DR. MURPHY.—At intervals they were typed, so we can say at any time in the study that at least 90 per cent were *Str. agalactiae*, and usually 95, so we speak here of *Str. agalactiae*.

DR. A. ASCOLI (New Brunswick, N. J.).—Your paper has been highly interesting, because you touch a problem to which we should pay more attention: that is, the interest of age and sex. You had a *Str. agalactiae* infection. They had experiments in Holland which show that mice through ingestion of estrogen become more resistant to *Str. agalactiae*, which illustrates that you can, I would say, immunize or produce higher resistance against pneumococci in mice also with estrogens. My question is this one: Do you say they can be kept alive by the action of hormones? How do they explain experimentally that with hormones you get a higher resistance? You will find as they grow older they become more susceptible to Streptococcus infection. I would like to hear your opinion about that.

DR. MURPHY.—I would like to slide around it if I may by saying that we only recognize the hormone hypothesis of Francis because it suggested that the change was taking place in the body that made them more susceptible. That is the only thought that ties this hormone theory to any of our work, and he didn't support his theory. It is really a pure hypothesis. I do not want anyone to get the impression that there is any evidence to connect hormones to this condition as of now.

DR. HENDERSHOTT.—Mr. Chairman, I am very much interested in this piece of research work. I think it is fundamental; I think that the author is to be complimented on doing a very profound piece of fundamental research in mastitis and, out of this discussion this morning, the thing that impresses me is this: If we are to have herds free from streptococcal infection, then we had better start to reduce the age of lactating animals in the herd. We have a better opportunity of controlling mastitis if we continually take out the older animals, replace them by home-grown replacements, and keep them in there for two, three, or four lactations.

As I get the picture here, the longer the lactation of the animal, that is, in numbers of years of lactation, the greater the possibility that the animal is foredoomed to have mastitis if she stays in the herd long enough. Is that true?

DR. MURPHY.—The indication is that it is merely a matter of time, that the difference between the individuals is the difference in the amount of time that they may avoid the condition. We are going to consider this other phase of it next in Chicago, I hope, but I would like to say right here that control programs usually recommend that animals discarded from a herd be replaced by first-calf heifers. Is that right?

DR. HENDERSHOTT.—That is right.

DR. MURPHY.—That has been done because such individuals are usually free of the infection, but if it works also to reduce the average age of the herd at the same time, it would work two ways.

DR. SWALES.—I would like to ask Dr. Murphy if he noticed any relation in incidence of mastitis in families of cows. Did you not have such an opportunity?

DR. MURPHY.—We have published a paper on resistance in families. We had two Guernsey families which are in this data you saw today; they disappeared in the mass of data. One family was resistant and one was susceptible. I would be very glad to send you a copy of it if you wish.

DR. HENDERSHOTT.—In connection with those two families, was this increasing-with-age pattern exhibited in those two families, or was it as constant as you have shown here this morning?

DR. MURPHY.—The families were very small, of course, but it was present. We felt at first that there was a resistance, an inherent resistance, and we attempted to get at it by using the records of all individuals, but we

‡ After the presentation of the paper, the following discussion was conducted.

couldn't do it because we have no correction factor for age that is usable on individuals. However, one family balanced the other in age. Therefore, age was no different between the two, and the difference in the susceptibility must have been inherited.

DR. J. V. McCAHON (Downingtown, Pa.).—Speaking from the standpoint of the home-owned herd, do you notice any correlation between the efficiency of breeding these cows and the infection or mastitis?

DR. MURPHY.—I am sorry, Dr. McCahon, it wasn't considered in the statistical analysis, and my opinion would be worthless.

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"Sworn to and subscribed before me this Thirtieth day of September, 1946:
Helen S. Bayless, Notary Public (commission expires October, 1949)."

Infection of the Bovine Udder With Yeastlike Fungi

JAMES M. MURPHY, V.M.D., and CHARLES H. DRAKE, Ph.D.

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WHILE SEVERAL SPECIES of bacteria and at least one species of Actinomyces have been found to invade the bovine udder and to reproduce therein, yeasts have been reported only once[6] and yeastlike fungi not at all.

Rolle[6] records the finding of 5 cases of udder inflammation caused by yeast in cows fed brewery byproducts and sour beet pulp. In the 2 cases he described in detail, 1 occurred in the third week and 1 in the third month of lactation. In each case, only one quarter was involved. There was swelling of the affected quarter, marked reduction in milk production, rise in body temperature and pulse rate, and macroscopic abnormality of the secretion. Recovery was spontaneous. Yeast in pure culture was recovered from each case and, while not identified, was described as forming round Micrococcus-like colonies which appeared darkly granular by transmitted light. There was no growth in agar stab culture. No difference was found between these yeasts and a strain of bread yeast. Inoculation of yeast from 1 of the cases into one quarter of a normal cow resulted in a case of mastitis similar to the natural one and from which the organisms were isolated in pure culture.

During a study of udder disease at the Dairy Research Station of the New Jersey Agricultural Experiment Station, infection of the bovine mammary gland with yeastlike fungi was observed. The information obtained in the study of these infections by the senior author, and in the study of the causative organisms by the junior author, is given below.

STUDIES OF INFECTIONS

Methods.—The cows included in this report were part of a herd of 120 purebred Guernsey and Holstein-Friesian cows maintained for experimental purposes. This herd has been under constant observation for udder infection and mastitis for a period of six years. The routine system of examinations, previously described[4], is briefly as follows: (*a*) bacteriologic examination of aseptically drawn colostrum obtained prior to calving, (*b*) bacteriologic and biochemical analysis of aseptically drawn, strict foremilk obtained at thirty to sixty-day intervals throughout the lactation period, (*c*) bacteriologic and biochemical analysis of aseptically drawn strict foremilk obtained from quarters daily when any new infection or any departure from the usual occurred, and (*d*) daily examination of the udder and secretion of every animal for swelling and gross abnormality, respectively.

All samples of strict foremilk represent the first 20 cc. of secretion removed from the gland with aseptic precautions. Routine cultural examination comprised plating 1 cc. of a 1 : 20 dilution of milk in 12 cc. of meat-infusion, blood agar. When necessary to detect small numbers of organisms, 1 cc. of secretion was plated directly. In addition, where fungi were present or suspected, the milk was plated in Sabouraud's agar containing lactose instead of maltose. For the culture of the fungi in fluid medium, Sabouraud's lactose broth was used.

RESULTS

Natural Infection.—Ten instances of infection of the bovine udder by yeastlike fungi have been observed during a six-year period. One occurred in 1938, 1 in 1939, 4 in 1940, none in 1941, 3 in 1942, and 1 in 1943. Data pertaining to the observed onset, the presence of the fungi in the milk, and the clinical symptoms evoked are presented in table 1.

Journal Series Paper of the New Jersey Agricultural Experiment Station, Rutgers University, Department of Dairy Husbandry.

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In 6 of the cases, the observed onset was clinical in that there was an inflammatory process evidenced by swelling of the gland or macroscopic abnormality of the secretion. In 5 of these cases, there was swelling of the gland lasting from three to seven days. In 1, no swelling was observed. In all of the 6 cases, there was macroscopic abnormality of the secretion lasting from six to twelve days. Fungi were present in the secretion for six to twenty-two days (average 11.1 days).

In 4 cases, the observed onset was routine in that the presence of the fungi was observed in samples of milk at a time when there was no clinical evidence of mastitis. Swelling of the infected gland occurred in only 1 of these cases and the duration was two days. Macroscopic abnormality of the secretion occurred in all but 1 of the 4 cases and lasted for three to eight days. Fungi were present in the secretion for nine to forty-eight days (average 29.5 days).

The results of the detailed examination of a representative case, the one that occurred in 1943, are shown in figure 1. The observed onset was clinical with swelling of the gland and macroscopic abnormality of the secretion. The former lasted five days and the latter eleven days. Fungi were present in the secretion in excess of 50,000 per cubic centimeter for the first three days and then began to decrease. The decrease continued, with three minor exceptions, until no fungi were present on the twenty-second day. Thereafter, no fungi were found in the secretion despite the fact that 1-cc. portions of strict foremilk were cultured up to the sixty-ninth day after the observed onset.

For the first eight days, the leucocyte content of the secretion was greater than 50 million. On the ninth day, the count began to decrease and, in general, continued to decrease until the count was less than one million on the nineteenth day. It should be noted that the leucocyte count rose slightly each time the fungus count showed a slight increase over the preceding day. After reaching a point below one million per cubic centimeter, the leucocyte count remained in the vicinity of one million for the duration of the observations, sixty-nine days from the observed onset.

Figure 2 shows the macroscopic appearance of strict foremilk samples for the first five days of the attack, and figure 3 shows the appearance of blood agar and lactose agar plates, 1 : 200 dilution after forty-eight hours' incubation at 37 C., for the fourth day of the attack.

Artificial Infection.—Cow 673, in the seventh month of the first lactation period,

TABLE 1—Summary of Data Obtained by the Observation of 10 Cases of Yeastlike Fungus Infection of the Bovine Udder

Cow no. and quar.	Observed Onset				Fungi		Clinical Observations	
	Type	Date	Lactation period	Week of lactation	Days present	Highest count	Swelling	Abnormal Milk*
H214 LF†	C.	8-14-38	5	27	8	45,500	7 da.	6 da.
497 RH	C.	6-13-39	3	10	9	80,600	5 da.	12 da.
H214 RF	R.	2-5-40	7	4	48	58,500	2 da.	3 da.
H307 RH	R.	4-8-40	4	2	30	2,120	None	None
557 RH	R.	8-3-40	1	50	9	49,400	None	8 da.
H380 LF	R.	11-15-40	2	34	31	28,600	None	4 da.
H363 LH	C.	1-19-42	3	27	15	11,180	None	7 da.
655 LH	C.	6-4-42	1	20	7	58,500	5 da.	8 da.
H452 RF	C.	10-15-42	2	8	6	Unknown	3 da.	7 da.
H482 RF	C.	1-5-43	1	29	21	137,000	5 da.	11 da.

* Macroscopic abnormality.
† LH = left hind quarter; LF, left front; RH, right hind; RF, right front.
C = Clinical.
R = Routine.

was selected as the subject of an artificial infection experiment. This animal was free of udder infection as shown by periodic, thirty-day, bacteriologic examinations in which no organisms other than Staphylococcus in numbers less than 200 per cubic centimeter or nonhemolytic udder diphtheroid bacilli in any number had ever been found. Leucocyte counts made at the same time had never exceeded 500,000 per cubic centimeter.

The fungus from the right front quarter of cow H482 was inoculated into the left front quarter of cow 673 three months after it had been isolated. During this time, the culture had been carried on Sabouraud's lactose agar. The inoculation was made by means of a sterile teat cannula which was dipped into a 1 : 400 dilution of a 24-hour broth culture in sterile physiologic salt solution. The cannula, estimated to carry not more than 2,000 fungus cells, was inserted several inches into the thoroughly cleaned teat of the experimental cow. The results of the bacteriologic examination and leucocyte count of strict foremilk, obtained prior to the afternoon milking, before and after artificial infection, are shown in figure 4.

The vertical broken line represents the point at which this case would probably have been recognized had it been the result of natural infection. It will be seen that the observations beyond this point are almost identical with those in figure 1. The observations prior to this point are most interesting. The inoculation occurred after the afternoon milking of "0" day. Strict foremilk was obtained prior to both the morning and afternoon milkings for the first five days after infection. Only the results of the examination of afternoon milk are shown in figure 4. No fungi were detected by the culturing of 1 cc. of strict foremilk from the morning or afternoon milking of the first day after infection. It was not until the morning milking of the second day that the fungi, four per cubic centimeter, were detected. The number of fungi present in the afternoon increased until there were more than 50,000 per cubic centimeter on the fifth day. As late as the afternoon of the fourth day after infection, the body temperature and the leucocyte content of the milk were still normal. The milk was not abnormal to the eye until the afternoon of the sixth day after the fungi had been placed in the teat.

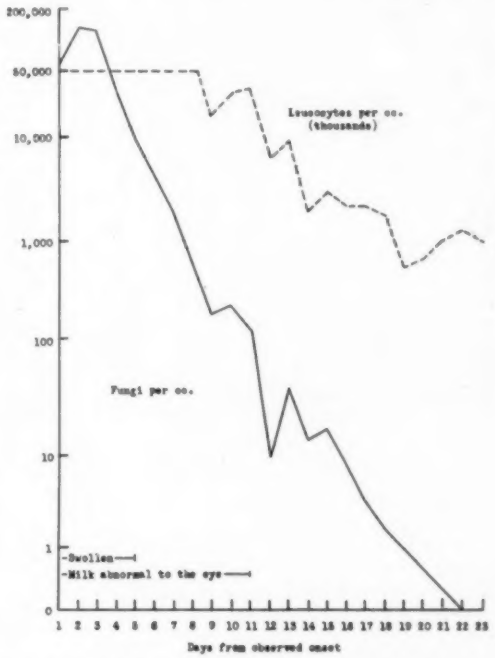

Fig. 1—Results of the examination of the udder and secretion of a case of natural infection (H482 RF).

CAUSATIVE ORGANISM

The term "yeast" has little taxonomic significance since it simply refers to an organism growing wholly or partly as budding cells or blastospores. Organisms of this type are known in all of the major groups of fungi, but most species are found in several genera of the fungi imperfecti or, if they form ascospores, in the Ascomycetes. Some fungi, such as Candida, the medical Monilia, form both mycelium and blastospores. The imperfect species with the latter characteristics are placed in the family Mycotoruloideae. The classification of this family has long been in a chaotic state, but the as yet unavailable monograph

on the group by Diddens and Lodder[2] should greatly clarify the problem.

Some fungi may form oidia or arthrospores by a fragmentation of the mycelium into unicellular components, as shown by the common dairy mold *Oöspora lactis* (*Geotrichum lactis*). When the oidia become shortened and rounded with age, it may be almost impossible to distinguish them from blastospores although the manner of formation is different for the two growth forms. Blastospores are formed by budding either from mycelium or from other unicellular elements. Oidia are formed by a fragmentation of mycelium into elongated cells with square ends, but usually become shortened and rounded at the ends so that they appear almost identical with blastospores which have ceased budding.

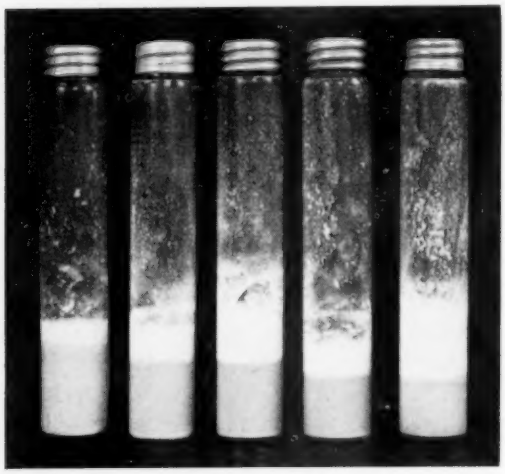

Fig. 2—Consecutive daily samples of strict foremilk (p. m.) from a case of natural infection (H482 RF) for five days following the observed onset.

Trichosporon is a poorly known genus of the Mycotoruloideae which shows all three growth forms: mycelium, blastospores, and oidia. Most of these organisms have been isolated from human infections either superficial and involving only the hair and skin or, more rarely, systemic infections. So far as can be determined, no infections in animals have been reported, although the junior author has had occasion to examine cultures of this genus isolated from dermatitis in cattle.

The organisms isolated by the senior author from the cases of mastitis clearly belong to the genus Trichosporon and probably are a new species. However, until the monograph of the Mycotoruloideae is available in this country, it would seem unwise to describe them as a new form, so that, for the present, only a description of the organisms will be given. In view of the fact that this is apparently the first report of such organisms from an animal source, it was felt highly desirable to compare the organisms with known cultures of Oöspora, Candida, and several species of Trichosporon: *Trichosporon malassezi*, *Trichosporon granulosum* (*cerebriforme*), and *Trichosporon cutaneum* (*asteroides*).

Cultures of the above species and of three strains of the organism isolated from mastitis, cases H452, 655, and H482, were grown as slide cultures on glucose broth, nutrient broth, Sabouraud's agar, nutrient agar, and corn meal agar. A slide of each fungus growing on each of the above mediums was removed at intervals, fixed, and stained by the method described by Martin *et al.*[3] Slide cultures of the Brown[1] type were also prepared and observed during growth. By these techniques, the manner of growth at different ages and on different mediums could be observed and the sequence of development readily followed. Measurements were made with an eyepiece micrometer of the living organisms in slide cultures and in drops of glucose broth cultures mounted under a coverslip. All of the measurements represent the range or average from at least 100 cells.

The organisms were also cultivated on glucose agar with potassium nitrate, ammonium sulfate, asparagine, or peptone as a nitrogen source and on Coon's liquid medium with the same nitrogen sources to obtain some information on the physiologic requirements. Glucose, sucrose and lactose broth, starch agar, nitrate agar, olive oil agar, blood agar, and bromcresol purple milk were also inoculated.

MORPHOLOGIC FINDINGS

In 24-hour slide cultures on glucose broth, the organisms isolated from mastitis were seen growing as blastospores and as mycelium. Blastospores were produced from the newly formed mycelium as both terminal and lateral budding cells. Some of the original single cells of the inoculum were seen forming mycelium while others were reproducing by budding to form clumps of blastospores. Occasionally, oidia were seen forming by fragmentation of the mycelium. Older cultures showed a sequence of development in which the mycelial stage predominated for a time, especially at the growing edge of the colony. The center of the colony, meanwhile, was replaced by unicellular forms. As the culture matured, at from forty-eight to seventy-two hours, the mycelium was almost entirely replaced by blastospores and oidia. With increased age, the oidia became shortened and rounded so that they were identical in appearance with blastospores, and the origin of the two forms could be determined only by following the development in younger colonies. Both the blastospores and the oidia became shortened, swollen, and vacuolated with age. On this medium, blastospore formation markedly predominated over oidia formation.

Glucose broth cultures of *T. cutaneum* and *T. malassezi* showed essentially the same sequence of development, but *T. granulosum* on the same medium showed only mycelium with no unicellular forms.

Nutrient broth cultures of the organism from mastitis showed a morphology similar to those on glucose broth except that oidia formation was more abundant and filaments of mycelium were found which showed fragmentation into a zigzag chain of oidia as was seen in *O. lactis*. The same was true of *T. cutaneum* and *T. malassezi* except that these organisms produced more oidia than blastospores. *T. granulosum* grew only as mycelium.

Sabouraud's agar slide cultures of the organism from mastitis showed abundant mycelial growth. Oidia were formed in abundance, but usually only a few blastospores were formed. Portions of the mycelium became tightly twisted or kinked so that when fragmentation occurred clumps of closely packed oidia were formed. As the cultures aged, there was a marked variability in the diameter of the hyphae.

Essentially the same development on Sabouraud's agar was shown by *T. cutaneum*

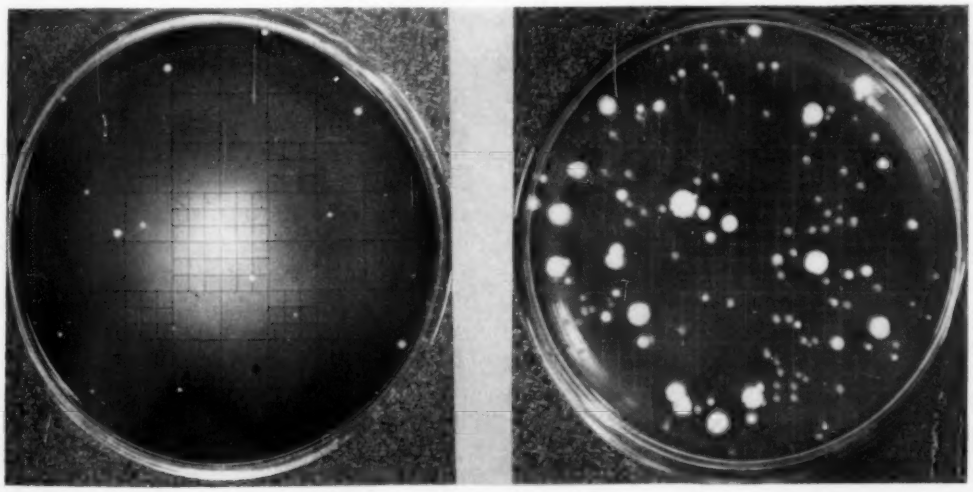

Fig. 3—The appearance of blood agar (left) and lactose agar (right) plates, 1 : 200 dilution, forty-eight hours' incubation at 37 C., prepared from strict foremilk on the fourth day of a case of natural infection (H482 RF).

and *T. malassezi*. The same peculiar twisting and kinking of the mycelium was seen as in the organisms from mastitis. *T. granulosum* differed only in that blastospores were apparently not formed, but oidia formation did occur.

Young, nutrient agar slide cultures of the organism from mastitis showed abundant mycelium with some fragmentation into oidia. In places, the mycelium was tightly twisted and kinked so that fragmentation gave rise to clumps of oidia. After forty-eight hours, blastospore formation was abundant and occurred both from mycelium and from some of the longer oidia. Where the mass of medium was sufficiently great the blastospores continued independent budding after separation from the mycelium and gave rise to whorls of blastospores. Most of the budding was unipolar.

Nutrient agar cultures of *T. cutaneum* and *T. malassezi* showed essentially the same growth pattern as the organisms from mastitis. However, *T. granulosum* showed only oidia and mycelium with few or no blastospores.

The morphology of the organism from mastitis on corn meal agar was characterized by the growth of mycelium which gave rise to abundant blastospores and some oidia. Blastospore formation predominated over oidia formation. However, the formation of oidia by fragmentation was very clear with zigzag chains of cells exactly duplicating oidia formation by *O. lactis*. As the cultures aged, the blastospores and oidia became thick-walled and rounded until cells of different origin were indistinguishable in morphology.

T. malassezi and *T. cutaneum* duplicated the appearance of the above cultures on corn meal agar. *T. granulosum* differed in that the mycelium was more abundant and persisted longer. Some blastospore formation took place as the cultures aged, but few, if any, oidia were formed. Most of the blastospores were formed by lateral budding from the mycelium.

The Trichosporon sp. isolated from mastitis had mycelium of variable diameter depending on the age of the culture. Measurements of 100 randomly selected hyphae from each of two different strains varied between 1.4 and 6.4μ with an average width of 3.2μ. Occasionally, swollen hyphae, which may represent chlamydospores, had diameters ranging up to 10μ.

The blastospores formed by the organism from mastitis were also variable in size and shape depending on age and stage of growth. Actively budding cells were usually elongated oval or pear-shaped but, with increased age, became shortened and often were almost completely rounded. The diameter of the blastospores varied from 2.9 to 7.15μ with an average diameter of 4.5μ. The length varied from 5 to 14μ with an average length of 8μ.

The oidia formed by Trichosporon sp. showed the greatest variation in dimensions, especially length, as might be expected from their manner of formation. Both long and short oidia were formed by hyphal fragmentation, and the longer cells usually underwent further fragmentation so that the average length did not have any great significance. The diameter varied from 2 to 5.7μ with an average width of 3.5μ. The length varied from 6 to 46μ with an average length of 19μ. When first formed, the oidia had squared-shaped ends, but as they matured the ends became rounded. Sometimes oidia produced blastospores by polar budding in such a way that they became shorter as budding progressed and the blastospores separated from the parent cell. With increased age, both oidia and blastospores became swollen, vacuolated, and developed thick walls. Vacuoles were also common in mycelium older than forty-eight hours, and their development can be traced from very small vacuoles which enlarge and coalesce.

PHYSIOLOGY AND CULTURAL CHARACTERISTICS

Only a few physiologic observations were made on the Trichosporon sp. from mastitis, and little is known of the physiology of the genus as a whole. Cultures on glucose and sucrose broth with bromcresol

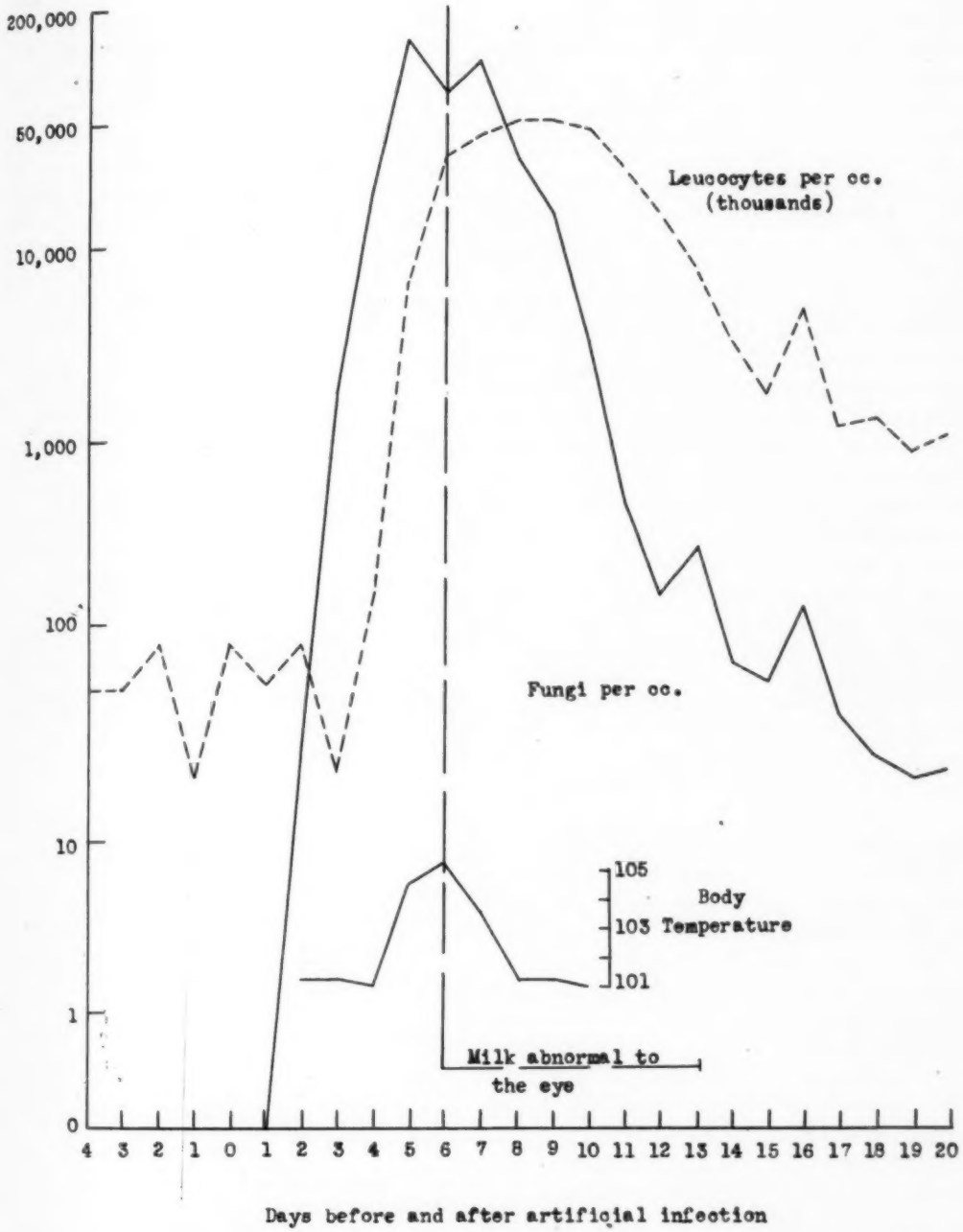

Fig. 4—Results of the examination of udder secretion and body temperature of a case of artificial infection (673 LF).

purple indicator showed slight acidity. A slight acidity developed more slowly in lactose broth. Strain 655 was especially slow in attacking lactose. Mannitol broth was rendered slightly alkaline as was the basal medium without added carbohydrate. Bromcresol purple milk was rendered slightly acid and coagulated, apparently by a rennet-like action. Starch was not attacked, and there was no hydrolysis of fats demonstrable on olive oil agar. Nitrates were not reduced. Human blood agar was discolored, but not hemolyzed, by the growth of the organism. The organism grew well at 37 C. and at room temperature, most rapidly at 37 C. There was slow growth at 20 C. and no growth occurred at 45 C. There was no growth under anaërobic conditions on any medium tested. The colonies appeared essentially the same on all mediums; moist, gray, at first with a slight tinge of yellow to brown in older colonies, and slightly rough and wrinkled. Older colonies often showed a sparse growth of white aërial mycelium. The colonies were round and raised above the medium; some extension of growth into the medium was observed. Trichosporon sp. was highly efficient in utilizing traces of nutrients, and there were sufficient impurities present in a distilled water, agar medium to support slight growth. A modified Coon's medium, containing chemically pure glucose and chemically pure mineral salts with no source of nitrogen, also supported very slight growth. By the use of suitable controls, it was demonstrated that, on a glucose agar medium, potassium nitrate would not serve as a source of nitrogen. Peptone was an excellent source of nitrogen and gave almost as good growth in the absence of glucose as with this carbohydrate present. Asparagine served as a very poor nitrogen source. Coon's liquid mineral salts medium, with 1 per cent glucose but with the nitrogen source eliminated, was also used to test the availability of various nitrogen sources, and the results duplicated those on the agar medium. Asparagine was a poor, and peptone a good, nitrogen source. The addition of glucose slightly improved the growth on the medium containing peptone but gave better stimulation of growth on the asparagine medium. However, the growth on asparagine glucose medium was not as heavy as on peptone alone. Potassium nitrate and ammonium sulfate would not serve as nitrogen sources except that one strain, 482, seemed to show slight growth on the ammonium sulfate glucose medium. However, in view of the extremely small amounts of impurities which would support growth, this result is open to question.

DISCUSSION AND CONCLUSIONS

Morphologically, the organism isolated from mastitis is a species of Trichosporon. The formation of mycelium, budding cells, and oidia observed in slide cultures rules out other possible genera. Confusion might arise with the genera Candida and Oöspora (Geotrichum) but, in the first genus, only mycelium and blastospores are formed while, in the latter genus, only mycelium and oidia are found. When compared on glucose broth, nutrient broth, Sabouraud's agar, nutrient agar, and corn meal agar with three known species of Trichosporon, the pattern of growth and the morphologic findings are well within the range of those of the three known species. This was shown by direct microscopic observation of growing cultures on Brown slides and by slide cultures fixed and stained at various stages of growth from ten hours to ten days.

Physiologically, the organisms show some characteristics different from other known species. Puntoni[5], in his monograph of the genus, states that neither acid nor gas is formed from carbohydrates, but the organism from mastitis showed slight acidity on glucose, sucrose, and more slowly on lactose. However, since the organism was an obligate aërobe, the acidity must have arisen from the incomplete oxidation of the carbohydrates such as is carried out by many fungi, and not from fermentation as in the case of most yeasts. The generic

diagnosis given by Puntoni[5] would have to be modified from the statement that "neither acid nor gas is formed" to "fermentation does not occur", using fermentation in the sense of anaërobic breakdown of energy sources.

In view of the animal and pathologic source of the organism and the formation of small amounts of acid from carbohydrates, this is probably a new species of Trichosporon, but a more exact comparison will have to be made with the species accepted in the monograph on the Mycotoruloideae by Diddens and Lodder[2] which is not, as yet, available in this country.

SUMMARY

Ten instances of infection of the bovine udder by yeastlike fungi have been observed during a six-year period in a herd of approximately 120 purebred Guernsey and Holstein-Friesian cows.

The infections were known to be present for from six to forty-eight days; the largest number of fungi per cubic centimeter was 137,000; 6 of the cases were attended by obvious swelling of the mammary gland for from two to seven days; and all but 1 was attended by macroscopic abnormality of the secretion for from three to twelve days.

The results of the examination of 1 case are given in detail. The causative organism in this case was inoculated into one quarter of a normal, uninfected udder with the result that the disease picture of the natural case was almost exactly reproduced.

The causative organisms from 3 cases were studied in detail. It was found that these organisms formed mycelium, blastospores, and oidia and, therefore, clearly belong to the genus Trichosporon of the family Mycotoruloideae.

In view of the animal and pathologic source of the organisms and certain differences in physiologic characteristics when compared to three known species of Trichosporon, it is probable that the organisms from mastitis represent a new species. A more exact comparison will have to be made, however, with accepted species when necessary information becomes available.

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Submucosal Glands in the Bovine Ileum

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THE CRYPTS of Lieberkühn are generally confined to the mucosa of the small intestine. As far as can be discoverd in the literature, the only glands found to date in the intestinal submucosa are the duodenal glands discovered in 1677 by Wepfer[1] and rediscovered by Brunner[2] (after whom they are called) in 1687.

In sectioning the ileum of an ox approximately 8 cm. above the ileo-cecal valve, numerous glands were observed in the submucosa, and especially within the lymphatic patches of Peyer. These glands consisted of a large chamber and numerous tubules. The tubules resembled the crypts of Lieberkühn, while the chamber was lined with the same kind of epithelium found on the surface of the villi. Certain of these ileac glands (fig. 1 and 2) seemed to be diverticulums of the intestinal lumen. Others, however, seemed to be inverted miniature intestines, being formed by the junction of numerous crypts of Lieberkühn which penetrated deeply into the submucosa. Figure 3 shows a gland of this type; in figure 4, we find two glands which illustrate this anatomic structure even better. Figure 4 is a low power picture of what appears to be an intestinal fold. In the center of the picture, we see a submucosal fibrous lamina, to the right and left of which we find the lymphoid tissue characteristic of the ileum. Only the left and right upper corners of this photomicrograph are occupied by the mucosa, i.e., by the tunica propria containing crypts of Lieberkühn. This is clearly delimited by a dark, narrow line representing the muscularis mucosae. Four submucosal glands can be seen in this small section, of which the two lower ones are most characteristic. They show glandular tubules coming from the muscularis mucosae and uniting to form a large, folded chamber deeper in the submucosa.

As many as 12 of these submucosal glands have been found in a single cross section of the ileum. This great number seems to indicate that they are normally present in this region.

SUMMARY

Numerous glands have been found in the submucosa of the ileum of the ox not far from the ileo-cecal valve.

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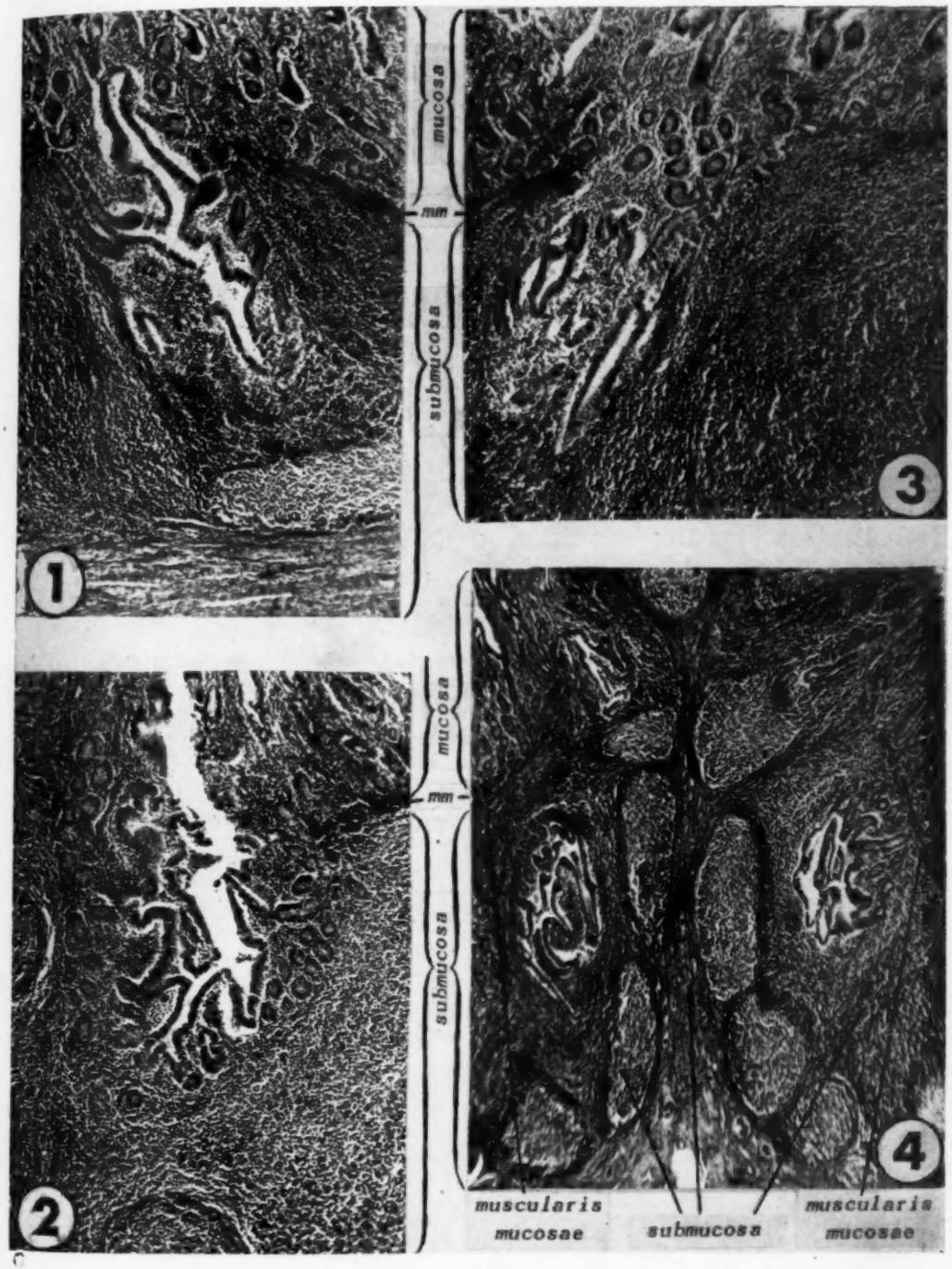

Fig. 1 and 2—Submucosal glands in the ileum of the ox; the main cavity of the glands is directly connected with the intestinal lumen.

Fig. 3—A submucosal gland in the ileum of the ox, the chamber of which arises from the union of several crypts of Lieberkühn which penetrate the muscularis mucosae.

Fig. 4—Four submucosal glands in the ileum of an ox; mm = muscularis mucosae.

Vaccination Studies on Bovine Trichomoniasis

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NUMEROUS investigators have demonstrated that cows which have recovered from bovine trichomoniasis were refractory to reinfection. The immunity produced by the initial infection varies in degree and duration. Very little information is available on whether it is possible to immunize cows against *Trichomonas foetus* by the intramuscular or intravenous injection of motile trichomonads. The purpose of this paper is to present experimental research on the vaccination of heifers for bovine trichomoniasis.

REVIEW OF THE LITERATURE

Kerr[1] injected a heifer intravenously; 15 million trichomonads were given as a first dose and the dosage increased during seven weeks to 500 million. This animal was mated with a bull which might have been infected at the time of service but in which trichomonad infection was never demonstrated. The service resulted in a normal pregnancy. Another heifer was given a series of intramuscular injections with *T. foetus*. She was exposed to the infection by the inoculation of a washed suspension of trichomonads into the vagina. The animal did not develop trichomoniasis, and a normal gestation period followed.

Garlick, Bartlett, and Hammond[4] attempted to transfer immunity passively from 3 immune cows to 4 heifers by repeated intravenous injections of serum or defibrinated blood. These injections appeared to protect the animals temporarily. Two of 3 treated heifers inoculated vaginally with *T. foetus* cultures did not become infected, while the 2 controls, not injected with immune serum or defibrinated blood, developed the infection. Two animals contracted trichomoniasis; one was bred naturally five months after the last serum injection by an infected bull, the other after vaginal inoculations with infected preputial flushings from a trichomonad-positive bull four days after the last serum had been given.

Morgan[2, 3] published, as abstracts, progress reports on part of the work presented in this paper.

MATERIALS AND METHODS

Thirty virgin heifers, all about 12 months of age, were injected with *T. foetus*, as given in detail in table 1. The heifers were divided into three groups of 10 each. In group A, 8 heifers were injected intramuscularly and 2 heifers intravenously approximately twice a week for 16 injections with 5 cc. of washed, bacteria-free trichomonads. The first injection contained 5 million organisms per cubic centimeter; the second, 7 million; third to sixth, 10; seventh and eighth, 15; ninth to eleventh, 20; twelfth, 30; thirteenth and fourteenth, 40; fifteenth and sixteenth, 50 million.

In group B, 10 heifers were injected intramuscularly twice a week for a period of three weeks with 5 cc. of washed trichomonads with a count of 50 million organisms per cubic centimeter. Group C received two intramuscular injections of 5 cc. each of living trichomonads (50 million organisms per cubic centimeter) during a period of three weeks.

Thirty virgin heifers which were not injected with trichomonads served as controls. All inoculated animals were bled and tested for trichomonad immobilization before injections were started and after each injection. The controls were bled at regular intervals. Tests, as described by Morgan[5], were run on all serums to determine their immobilization titer.

All of the animals used in these experiments were tested once a month and were negative for brucellosis. Red and white cell counts and hemoglobin values were determined at regular intervals, and all were well within the normal range throughout the experiment. Temperatures taken at various times did not show any deviation from the normal. The animals also gave negative reactions to the tuberculin test. At no time until the day of inoculation into the vagina and uterus

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were trichomonads isolated from the genital tract of any heifer, although prior weekly examinations were made.

T. foetus used in this work was a strain isolated by Morgan and Wisnicky[6] and subsequently studied by Morgan[7]. Forty-eight to 96-hour cultures were used, as the maximum growth occurred at this time. The trichomonads were centrifuged and washed four times with 0.7 per cent saline solution and then diluted to the proper concentration for injection purposes, inoculations of the genital tract, or as antigen for the immobilization test. The dilution counts were made with a hemacytometer.

TABLE 1—Injection Schedule for Heifers Vaccinated with Motile Trichomonas Foetus

Group	No. of heifers	Method of injection	Length of time of injections	No. injections	Amt. injected (cc.)	No. organisms /cc. (millions)	Maximal immobilization titer
A	8	Im.	2 mo.	16	5	5-50	1 : 256
A	2	Iv.	2 mo.	16	5	5-50	1 : 256
B	10	Im.	3 wk.	6	5	50	1 : 32
C	10	Im.	3 wk.	2	5	50	1 : 8
Controls	30	..	0	0	0	0	0

Im. = intramuscular.
Iv. = intravenous.

At the first heat period after the last injection of trichomonads, each heifer was artificially inseminated with fresh, normal semen. Twenty-four hours later each was inoculated in each uterine horn with 10 cc. of motile *T. foetus* with a concentration of 50 million organisms per cubic centimeter. Results are shown in table 2.

RESULTS

Group A.—Five heifers conceived on the first insemination, 2 on the second, and 2 on the third. All gave birth to normal calves. The remaining heifer developed a mild case of rickets and was given a short breeding rest, but this animal conceived on the fourth insemination and calved normally. The 10 control animals developed varying degrees of trichomonad vaginitis, endometritis, and pyometra. One heifer produced a normal calf after a severe case of pyometra. Trichomonads were isolated at various intervals from the controls; however, the organisms could be found in only 1 vaccinated heifer.

Group B.—Of the vaccinated animals in group B, 7 showed heavy trichomonad infections and the 3 negative animals became pregnant after the first insemination. Only one insemination per animal was performed on this group as the majority were discharging a purulent exudate containing trichomonads and were sent to slaughter. In the 10 noninjected control heifers, 6 were positive for *T. foetus*, of which 5 showed no evidence of pregnancy while 1 carried a full-term calf. Of the 4 negative heifers, 3 became pregnant and 1 failed to show evidence of pregnancy.

Group C.—Eight out of the 10 vaccinated animals in this group showed heavy trichomonad infections while 2 were negative for *T. foetus* and did not become recogniz-

TABLE 2—Results of Vaccination Studies on Bovine Trichomoniasis

Group A			Group B			Group C		
Cow No.	No. days infection	Result of insemination	Cow No.	No. days infection	Result of insemination	Cow No.	No. days infection	Result of insemination
Vaccinated								
1	N.	N.P.	1	N.	N.P.	1	45	N.C.
2	N.	N.P.	2	41	N.C.	2	N.	N.C.
3	N.	N.P.	3	22	N.C.	3	N.	N.C.
4	N.	N.P.	4	26	N.C.	4	37	N.C.
5	N.	N.P.	5	38	N.C.	5	18	N.C.
6	N.	N.P.	6	46	N.C.	6	33	N.P.
7	4	N.P.	7	33	N.C.	7	64	N.C.
8	N.	N.P.	8	27	N.C.	8	48	N.P.
9	N.	N.P.	9	N.	N.P.	9	47	N.C.
10	N.	N.P.	10	N.	N.P.	10	76	N.C.
Controls								
1	46	N.C.*	1	N.	N.P.	1	N.	N.P.
2	39	N.C.	2	N.	N.C.	2	N.	N.C.
3	66	Pyometra in left horn—N.P. in right.	3	83	N.P.	3	46	N.P.
4	39	N.C.	4	16	N.C.	4	49	N.C.
5	28	N.C.	5	N.	N.P.	5	N.	N.P.
6	61	N.C.	6	N.	N.P.	6	N.	N.P.
7	54	N.C.	7	33	N.C.	7	N.	N.P.
8	24	N.C.	8	19	N.C.	8	48	N.C.
9	30	N.C.	9	25	N.C.	9	N.	N.P.
10	32	N.C.	10	35	N.C.	10	45	N.P.

*No evidence of pregnancy. N. = negative.
N.C. = no conception. N.P. = normal pregnancy.

ably pregnant. In the control group, 4 animals were positive for *T. foetus*, and 2 of these became pregnant. Of the 6 animals which were negative, 5 became pregnant and 1 did not. Since these animals showed the various manifestations of trichomoniasis, they were sent to slaughter two to three months after the first service.

SUMMARY AND CONCLUSIONS

Group A.—Nine out of 10 heifers were apparently protected temporarily against trichomoniasis by a series of 16 intramuscular or intravenous injections of living organisms at approximately semiweekly intervals. All of the control animals which were not vaccinated became infected to some degree with *Trichomonas foetus*. At the first heat period after the last injection, each female was artificially inseminated with fresh, normal semen. Twenty-four hours later each was inoculated in each uterine horn with 10 cc. of motile trichomonads. The immobilization titer of the injected animals ranged from 1 : 64 to 1 : 256. All vaccinated animals conceived after one to four inseminations and gave birth to normal calves. Only 1 control became recognizably pregnant; it carried a trichomonad infection in the opposite horn for some time.

Group B.—Seven animals out of 10, after receiving six injections over a period of three weeks, became infected with trichomoniasis. The 3 animals which failed to contract the infection became pregnant. In the noninjected controls, 6 heifers were positive for *T. foetus* of which 5 gave no evidence of pregnancy and 1 became pregnant. Three of the 4 negative heifers conceived. Six injections of *T. foetus* intramuscularly over a three-week period failed to protect the animals from a trichomonad infection. Immobilization titers of the injected animals ranged from 1 : 4 to 1 : 16.

Group C.—Two injections of trichomonads over a three-week period failed to protect 8 out of 10 heifers from trichomoniasis when inoculated *in utero* with motile trichomonads. The 2 negative animals did not become recognizably pregnant. In the controls, 4 animals were positive for *T. foetus*. Two of these animals became pregnant. Five out of the 6 negative heifers conceived. The immobilization titers of the vaccinated animals ranged from 1 : 4 to 1 : 8. There appeared to be no correlation between the immobilization titer and the degree of protection. Breeding females recovering from trichomoniasis and those temporarily immune to reinfection showed no immobilization titers. Also, females infected naturally showed no titers during the peak of the infection. From the data presented, with 30 vaccinated virgin heifers and 30 control virgin heifers, it appears that in the series of 10 animals injected intramuscularly or intravenously 16 times at approximately semiweekly intervals, with living trichomonads, temporary immunity was produced against *T. foetus*. The next two series of 20 animals injected intramuscularly with living *T. foetus*, either two or six times during a three-week period, were not protected against *T. foetus* infections.

Although temporary immunity may result after a long series of injections, the vaccination of heifers is ineffective for protecting breeding females and cannot be recommended as a practical procedure.

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Some Experimental Investigations on the Etiology of Leucemia in Cattle

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THE ETIOLOGY of leucemia in cattle and other mammals remains obscure. Dobberstein and Paarmann[1] suggest that a chronic poisoning with protein may play an etiologic rôle in the leucemic disorders of cattle and that chronic protein poisoning may release a latent infection with an unknown virus in the diseased animals.

In this connection, Siegmund[2] has shown that a parenteral inoculation of protein involves an expansion of the active mesenchymal tissue and a lymphocytic proliferation. Pentimalli[3] has shown that chronic protein poisoning in rabbits is accompanied by alterations in the hematopoietic tissue very similar to those in lymphatic leucemia. Bungeler[4] showed that indol poisoning in mice gives rise to alterations similar to those in lymphatic and myelogenous leucemia. Charles Grégoire[5] showed that reinjection of the sensitizing agents in sensitized animals causes edema and hyperplasia of the lymphatic and reticulo-endothelial tissue. Vaubel[6] has confirmed the findings of Pentimalli, and Apitz states that the principal alterations in rabbits allergic to horse serum are proliferations of cells in the pulp of the spleen. R. Knepper[7] leared that allergic alterations of the tissue can be influenced by autocytotoxins.

On the basis of these investigations and suppositions, a study has been made of the reaction of cattle to parenteral chronic protein poisoning. The experiments were carried out on 10 calves, 5 females and 5 males, which weighed from 95 to 100 kg. at the beginning of the experiment.

The state of the animals' health was investigated by observation of the calves fourteen days before the experiment was begun. During the whole experimental period, the calves were clinically examined daily and, once a week, hematologic examinations were made by means of the usual methods. During the first seven days of the experiment, each calf was intravenously injected daily with 50 cc. of skimmilk, in the following two weeks with 100 cc., and in the remaining time with 200 cc. During the whole experimental period, 2 calves also received an intravenous injection of Streptococcus culture suspended in physiologic salt solution once a week. Further, 2 calves received twice weekly, for two weeks, 20 cc. of suspended spleen tissue, intraperitoneally.

After slaughtering the calves, histologic examinations were made of bone marrow, spleen, lymph nodes, kidney, and heart. Two of the calves died from enteritis and phlebitis, respectively. The remaining 8 lived throughout the whole experimental period.

Subsequent to each injection of skimmilk, in the first three weeks of the experiment, symptoms of shock occurred, and each injection was followed by a period of weakness but, apart from this, the calves did not show any trouble. The clinical evidence noted was monotonous and can be summarized.

Calf A-5163 received a total of 39,950 cc. of skimmilk, intravenously; calf A-5168, 29,750 cc.; calf 5166, 26,150 cc.; and calf 5171, 25,350 cc. An increase in the size of the superficial lymph nodes was noticed in all the animals. The enlargement was first noted in A-5163 and A-5168, three weeks after the experiment was started. In the following weeks, the increase in size continued and the maximum was reached after eighteen to twenty

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weeks, after which a decrease in size occurred. When greatest, the superficial lymph nodes were twice the size of corresponding lymph nodes in untreated, healthy calves of the same age. Besides the increase in size of the superficial lymph nodes, a continual emaciation occurred although the appetite was not exceptional.

Hematologic examination revealed a decreasing number of erythrocytes throughout the period of the experiment and, at the end of this time, the number was only half of that at the beginning. Morphologically, in the increased number of erythroblasts, there was seen a cell type occurring rarely in healthy calves of that age. In the later experimental period, the proportion of erythroblasts: leucocytes was found to be as 2:100, and anisocytosis and polychromasia were noted.

In the first two weeks, the number of leucocytes increased. Then there occurred a decrease followed for four to six weeks with another increase. The second increase was succeeded by a decrease lasting three to five weeks, which was succeeded again by an even increase lasting to the end of the experiment. The greatest number of leucocytes (47,000) was seen in A-5163, which was four to five times the number occurring in healthy calves. Morphologic examination revealed the lymphocytes to be the predominant type of white blood cells. In some weeks, the total number of leucocytes was four times higher than in normal calves. Lymphoblasts occurred singly only. Numerous pathologic cell types and juvenile lymphocytes occurred commonly, but a picture identical to those of lymphatic leucemia was never noted. The total number of granulocytes was markedly less increased than that of lymphocytes.

Postmortem examination revealed uniform gross lesions. In A-5166 and A-5171, the superficial cervical lymph nodes were twice normal size, in A-5163 and A-5168, 1.5 times the normal size. In A-5171, the hepatic nodes were twice the size of the corresponding ones in healthy calves of the same age and, in A-5166, they were as large as a hen egg. In the other 2 calves, the size of the hepatic lymph nodes was unaltered. The posterior mediastinal nodes in A-5171 were 15 by 6 by 4 cm.; in A-5166, 22 by 9 by 5 cm.; in A-5163, 12 by 5 by 4 cm.; and in A-5168, 10 by 4 by 3 cm. In A-5171, the spleen was 1.5 times the size of spleens in healthy calves; in the other 3 calves, the spleen was unaltered. The borders of the spleen were rounded in all the calves. Numerous greyish spots as large as the head of a pin occurred over the whole cut surface. No gross lesions were seen in the lungs, heart, and liver. In the kidneys of all the animals, miliary to supermiliary, greyish foci were present. In all the animals, the bone marrow of the humerus was largely fat marrow; active red marrow was seen only as a narrow sheath around the yellow marrow and in the proximal epiphysis.

Histologic examination of the lymph nodes of A-5166, A-5163, A-5168, and A-5171 revealed a lymphoid parenchymatous tissue very poor in cells. Germ centers were less numerous than in normal lymphoid parenchyma. Extensive areas consisted of a reticular tissue very poor in cells and, as a rule, without trace of germ centers. Apparently, the capsule, the trabeculae, and the greater blood vessels were unaltered.

At higher magnification, the parenchyma poor in cells showed the different cellular elements to be more distinctly separated than normally. Most of the cells proved to be lymphocytes, but plasm cells occurred also, as a rule, in small nests. Lymphoid cells similar to so-called lymphoblasts occurred singly. Mitosis was seldom seen. In the few germ centers, cell types similar to those in normal tissue were found. Throughout the whole tissue, numerous cells undergoing degenerative changes of the nucleus were noted.

In the parts very poor in cells, a typical reticular tissue occurred, with reticulocytes as the predominant cell type. Scattered among these were lymphocytes and plasmocytes, but in small numbers only. In addition, the tissue was characterized by great

numbers of vascular elements, mostly lymphatic vessels filled out with lymphocytes. Lesser blood vessels were not numerous. Now and then, mitosis was noted in reticulocytes. Cells showing degenerative changes of the nucleus were present, as in the cell-rich parts of the tissue.

The endothelial cells of the capillaries were unchanged. Now and then, a single hypertrophic cell was seen, however. Here and there, the small arterioles were surrounded by lymphocytes. Mainly, the greater blood vessels were unchanged but, now and then, small infiltrations with round cells were found in the adventitious connective tissue.

The histologic structure of the spleen was unaltered. As a rule, the malpighian bodies were distinctly outlined The larger blood vessels behaved as in the lymph nodes. The red pulp was relatively poor in cells with lymphocytes most numerous. Plasmocytes appeared in greater number than normally, most frequently in small nests, The reticulocytes were, in some places, unaltered—in others, hypertrophied.

The bone marrow was very poor in cellular elements, with fat cells predominating. Here and there were small nests composed of primitive cells. Erythropoietic tissue seldom occurred.

In the lungs and heart, apart from vascular alterations similar to those in the lymph nodes, no important changes were noted.

The structure of the liver was unaltered. The intralobular capillaries were ectatic and filled with lymphocytes. Kupffer cells were hypertrophied.

In calves which, in addition to inoculation of skimmilk, were twice treated intraperitoneally with spleen tissue, the blood pictures were identical with those in calves treated with skimmilk only. The lymphoid parenchyma was richer in cells than in the calves described above, and germ centers were more numerous.

The calves treated with both skimmilk and streptococci, intravenously, showed a blood picture differing strongly from those of the calves above mentioned, as juvenile neutrophilic cells (metamyelocytes) were seen in great numbers.

The histologic structure of the lymph nodes, spleen, heart, and kidney were identical to those previously described. The liver and bone marrow, on the contrary, showed alterations totally different from these. The bone marrow contained numerous cells, but the great number of cell accumulations was the most conspicuous difference. The architecture of these accumulations was identical with malpighian bodies in the spleen. Not a trace of myeloid and erythropoietic cellular elements were seen in them. There were great infiltrations of lymphocytes in the blood vessels of the liver, and also numerous ones intralobularly.

Large amounts of skimmilk inoculated intravenously into calves gave rise to serious anemia and reduction of the erythropoietic tissue of the bone marrow. A simultaneous intravenous inoculation of streptococci or intraperitoneal inoculation of spleen tissue did not influence the reaction.

The leucocytes of the peripheral blood ran a wavelike curve. The differential count showed mononucleosis due to increase in the numbers of lymphocytes. Polynucleosis occurred due to extravenous deposit of the skimmilk, but disappeared as soon as the abscesses which developed had been lanced. The mononucleosis represented the specific reaction to parenteral inoculation of skimmilk. This reaction was not influenced by intraperitoneal inoculation of spleen tissue On the contrary, the reaction changed when streptococci were simultaneously inoculated, intravenously.

Among the lymphocytes, numerous pathologic cells were found. Frequently, pathologic cells were more numerous than normal lymphocytes, and juvenile lymphocytes were numerous in the peripheral blood. The investigations proved that intravenous inoculation of skimmilk in calves gave rise to a toxic lymphocytosis, which was not identical to those characteristic of lymphatic leucemia.

The clinical symptoms are identical to

those described in animals sensitized by Pentimalli[3] and Grégoire[5]. The main clincal picture was enlargement of the lymph nodes during the first twenty weeks, followed by a decrease in size.

The most remarkable histologic changes occurred in the lymph nodes, which inferred corresponding changes in the spleen, bone marrow, and liver.

The most conspicuous histologic observations were a violent decrease in number of parenchymatous cells in the lymph nodes and the frequency of degenerated cells. The parenchyma in the lymph nodes was, to a large extent, functionally inactivated and rarely showed mitosis. There were great areas of lymphoid parenchyma poor in cells and with very few germ centers. The reaction proved that intravenous inoculation of large amounts of skimmilk in calves gave rise to an emptying of lymphocytes from the lymphoid parenchyma and depression of functional activity. Besides, the cells of the reticulo-endothelial system showed distinct proliferation principally conspicuous in the lymphoid parenchyma and the bone marrow, the parts of the lymphoid parenchyma poor in cellular elements. These areas were highly similar to the primitive stages of developing embryonic mesenchymal tissue. The reaction can, in no respect, be identified with histologic changes occurring in leucemia. On the contrary, the reaction is identical to the allergic reaction in lymphoid tissue described by Grégoire[5].

A peculiar reaction was noted in the bone marrow of calf A-5170, where nests of lymphoid cells were numerous in the bone marrow of the humerus. As is known, small nests of lymphoid tissue may sometimes occur in the bone marrow. They seldom attain the extent seen in this calf. But even if the peculiar observation of A-5170 is counted, the reaction of the lymphoid tissue was completely different from changes occurring in lymphatic leucemia.

The hyperplastic reaction noticed in the reticulo-endothelial elements was entirely different from those found in so-called reticulo-endotheliosis. Moreover, the intralobular myeloid cell nests in the liver of A-5170 were completely different from those occurring in myeloid leucemia, but were identical to the heteroplastic myeloid neoplasms occurring in infectious diseases. In this calf, the reaction must be due to two different reasons: (1) a violent inactivation of the lymphoid parenchyma due to inoculation of skimmilk and, (2) a substituting reaction to the insufficient bone marrow due to inoculation of streptococci.

The results of these experiments are: Intravenous inoculation of skimmilk in great doses gives rise, in calves, to a violent lymphocytosis, degenerative changes throughout the lymphoid parenchyma, functional inactivation of great areas of the lymphoid tissue coupled with transformation of this to a tissue greatly resembling developing juvenile mesenchymal tissue, decrease of the functional properties of myeloid tissue, and activation of the cellular elements of the reticulo-endothelial system. The alterations are in contradiction to those noticed by Pentimalli and Bungeler[7], which are said to be identical to leucemic changes; the changes on the contrary, are completely analogous to those described by Grégoire[5] as allergic reactions in sensitized tissue. The experiments have by no means confirmed the theory that leucemia in cattle is caused partly by chronic protein poisoning.

As distinct from other animals (mice, fowl) until now, transference of leucemia from one cow to another has failed even when homologous animals were used (Engelbreth-Holm and Plum[8]). However, Dobberstein thinks that leucemia in cattle is caused by an unknown virus released by chronic protein poisoning. The justice of this theory I have put to the test in calves which for four to six weeks were given skimmilk intravenously in the manner described and then simultaneously with the skimmilk inoculations treated intravenously—in group 1, with serum originating from 2 cows suffering from leucemia; in group 2, with fluid from the lymph nodes of a cow suffering with leucemia; and, in group 3, with pulp from the spleen of a leucemic cow. Further, I tried to trans-

plant the disease to healthy calves untreated with skimmilk by means of inoculation of lymphoid tissue originating from lymph nodes of a cow affected with leucemia.

The experiments with intravenously inoculated skimmilk reveal that those did not give rise to development of leucemia in calves. If, therefore, leucemia should develop in calves of the first three groups but not in the last, the cause would, in all probability, be a combined effect of the protein poisoning and a virus enclosed in the material originating from the leucemic cows. The inoculated leucemic material was poor in cells—not cell-free. This is, however, without significance as the results obtained indicate.

The experiments were carried out on calves at the same age as those used in the protein poisoning tests previously described.

Four calves were inoculated, intravenously, during four and a half weeks with skimmilk as described. Simultaneously, they received twice weekly for two weeks 250 cc. of serum originating from 2 leucemic cows. Then the calves were observed for four and a half months. The inoculation of skimmilk ceased after the last treatment with leucemic serum. Clinically and hematologically, the calves reacted the same as calves getting skimmilk only, intravenously. The enlargement of the superficial lymph nodes disappeared gradually in the course of six weeks and, after that lapse of time, no change of the blood could be noted. After the calves had been slaughtered, no pathologic changes could be demonstrated in the lymph nodes, spleen, liver, and bone marrow, nor did histologic examination of the organs reveal pathologic alterations. The result of the experiment is that simultaneous, intravenous inoculation of skimmilk and leucemia serum did not give rise to leucemia in calves suffering from chronic protein poisoning.

Two other calves received, throughout six weeks, daily intravenous inoculations of skimmilk; then they recieved 15 cc. of fluid of leucemic lymph nodes subcutaneously. The inoculation of the fluid from the leucemic lymph nodes was immediately followed by a great enlargement of the lymph nodes draining that part of the body. The enlargement persisted for four weeks, then gradually decreased in size. The enlarged nodes showed no sign of inflammation.

Hematologic and postmortem examination revealed no pathologic alteration. Especially, no changes identical to those occurring in leucemic cattle.

Five weeks after 2 calves had received skimmilk intravenously, they reacted, subcutaneously, to 20 cc. of pulp from the spleen of a leucemic cow. The reaction did not differ from those noticed in calves inoculated with fluid from leucemic lymph nodes.

The results obtained in these experiments suggest (1) a problematic, unknown virus (Dobberstein) of leucemia in cattle did not give rise to leucemia in calves sensitized by daily intravenous inoculations of skimmilk through several weeks, and (2) the reaction caused by inoculation of cell-poor substances originating from leucemic tissue is unspecific and in no way identical to leucemic changes.

Attempts were made to transfer leucemia by means of inoculation of tissue originating from leucemic lymph nodes. The inoculation was made on 4 calves which differed from the previous ones in that they had not been treated intravenously with skimmilk. Each calf received 20 cc. of leucemic tissue subcutaneously. The inoculation in 2 calves was followed by inflammation, but in the remaining 2 calves, it did not give rise to any sign of inflammation. In the last 2 calves, in the course of the first seven days after inoculation, a small swelling at the place of inoculation was noted. The regional lymph nodes did react by a small swelling of but few days duration. In the ensuing six weeks, the clinical picture remained unaltered; then there was a gradual enlargement of the regional lymph nodes. In 1 of the calves, this remained four months after the inoculation; then the lymph nodes diminished in size and, upon slaughter seven months after inoculation, the lymph nodes were

normal. Neither gross nor microscopic lesions characteristic of leucemia could be seen in lymph nodes, spleen, liver, and bone marrow.

In the fourth calf, the reaction passed away as in the foregoing one, but the enlargement of the lymph nodes had not stopped after four months; their size continued to augment, and seven months after inoculation, their size was twice that of corresponding normal lymph nodes on the other half of the body. In the next two months, the size of the lymph nodes diminished gradually, and at slaughter, nine months after inoculation, the regional lymph nodes as well as the other lymph nodes of the body were the size of lymph nodes of healthy calves the same age. Spleen, liver, and bone marrow did not contain gross lesions characteristic of leucemia, nor could such be found by microscopic examination of the organs.

The results obtained are: a massive subcutaneous inoculation of live tissue of leucemic lymph nodes did not give rise to leucemic changes in healthy calves.

It is unlikely that leucemia in cattle is caused by a specific virus, for even when the resistance of the calves used in the experiments was broken down by a chronic poisoning by means of intravenous inoculations of skimmilk, it did not give rise to leucemia by means of a material poor in cells. The possibility persists that the virus is coupled with cells and, therefore, it cannot be denied that the content of virus in the different fluids inoculated was too small to cause the disease. This possibility probably can be excluded also as the transference did not succeed following the inoculation of massive doses of material from leucemic tissue rich in cells.

It has been supposed that the soil, water, and heredity may play a rôle in the etiology of leucemia in cattle. The investigations carried out by the author seem to exclude soil and water as etiologic factors since, of the cows examined, 1 originated from the northwestern part, 2 from the southwestern part, 4 from the southern part, and 1 from the eastern part of Denmark, 1 from the northern part of Seeland, and 3 from Jutland. In other words, the cows suffering with leucemia originated from different parts of Denmark, each with an entirely different soil. Hence, leucemia in Danish cattle must be looked upon as a disease appearing without connection to specific soil.

It cannot be denied, however, that the disease occurs in some peculiar places. Especially in the better herds, the disease can occur constantly. In such herds, it is natural to conclude that hereditary factors play a rôle. However, it is very difficult to obtain facts on hereditary factors as in these herds the animals are related, but suggestions of a hereditary causal factor may emerge even if careful investigations by means of modern genetic methods revealed that heredity can be excluded. However, the observations here made are too insignificant to elucidate this unsolved question in man and cattle.

Earlier, there were reported additional results of blood examinations in leucemic cows. But even if more authors have published results of blood examinations carried out in healthy cattle from districts having constant leucemia, or if some authors suppose that animals not showing clinical leucemia can still show blood changes which should be interpreted as an expression that the particular animal is, or later will be, attacked by clinically manifest leucemia, these examinations have proved to be a very doubtful basis for combating the disease.

In order to examine the problem, I have, over a period of three years, carried out systematic hematologic examinations in herds with constant leucemia. The blood of all animals more than 3 years old has been examined twice a year. A total of 596 animals has been examined. In 110 of them, the examinations have been carried out more than once, and owing to the possibility of comparison, these 110 animals are a very valuable part of the material presented. In order to procure parallel material, there has been carried out also a

single hematologic examination in herds free of leucemia.

The term lymphocyte has been used only for cells identical to healthy blood lymphocytes. The term pathologic mononuclear cell has been used to designate cells characterized morphologically by: a size of 15 to 25μ, a relatively broad cytoplasm staining faintly and having an eccentric round or oval nucleus filling about half of the cytoplasm, a thin, smooth, and distinct nuclear membrane, the chromatin occurring as a delicate network containing chromatin fragments of varying size and shape, in some few juvenile cells nucleoli occurring in a number of from one to six or eight.

The healthy herds contained 57 cows over 2 years old. The hematologic examination showed that: less than 7,000 leucocytes in 1 mm.^{3} of blood occurred in 9 animals or 15 per cent; from 7,000 to 9,000 occurred in 12 animals or 21 per cent; from 9,000 to 10,000 occurred in 12 animals or 21 per cent; from 10,000 to 12,000 occurred in 24 animals or 43 per cent; and more than 12,000 occurred in no animals.

Less than 50 per cent lymphocytes occurred in 21 animals or 36.9 per cent; from 50 to 59 per cent occurred in 36 animals or 63.1 per cent; over 60 per cent occurred in no animals.

Less than 60 per cent mononuclear cells occurred in 46 animals or 80.7 per cent; from 60 to 65 per cent occurred in 11 animals or 19.3 per cent; more than 65 per cent occurred in no animals. Nor did pathologic mononuclear cells occur.

This examination showed that an occasional hematologic examination of a leucemia-free herd revealed only a normal number of leucocytes, and that the relative number of mononuclear white blood cells never exceeded 65 per cent. Typical lymphocytes and monocytes were the only mononuclear cells—a finding corresponding with the statements of other authors.

In herds with contant leucemia cases, there occurred: less than 8,000 leucocytes in 1 mm.^{3} of blood in 122 animals or 20.4 per cent; from 8,000 to 9,000 in 118 animals or 20 per cent; from 9,000 to 10,000 in 85 animals or 14.2 per cent; from 10,000 to 12,000 in 89 animals or 15 per cent; from 12,000 to 15,000 in 71 animals or 12 per cent; and more than 15,000 in 110 animals or 18.4 per cent.

Among animals with more than 12,000 leucocytes in 1 mm.^{3} of blood, the mononuclears exceeded 65 per cent more than once in 37 animals or 20.3 per cent, and among animals with more than 15,000 leucocytes in 1 mm.^{3} of blood more than once in 21 animals or 19.9 per cent. Compared with herds free of leucemia it is significant that a great number of animals belonging to herds with constant leucemia show leucocytosis. It must be remembered, however, that a single demonstration of leucocytosis by an occasional blood test is significant only as more physiologic and pathologic circumstances can give rise to leucocytosis. But as 20.3 per cent of the animals have been found leucocytic by repeated examinations carried out at intervals of half a year, and as 19.9 per cent of the animals constantly had more than 15,000 leucocytes in the peripheral blood without simultaneous clinically discernible leucocytosis, it must be doubtful that accidental physiologic or pathologic causes can be the causal factor of this leucocytosis. It must be more obvious to assume that in these cases a peculiar labile activity in the hematopoietic tissues of the animals is responsible for the leucocytosis. It is significant to note also that 4 out of 7 animals which later became leucemic belonged to the group of 21 animals constantly showing more than 15,000 leucocytes in 1 mm.^{3} of blood. In other words, 19 per cent of these 21 animals were later attacked by leucemia.

Morphologic examination showed that more than 90 per cent of all lymphocytes were completely normal in 11 animals or 1.9 per cent; from 80 to 89 per cent in 50 animals or 8.3 per cent; from 70 to 79 per cent in 118 animals or 19.3 per cent; from 60 to 69 per cent in 166 animals or 27.8 per cent; and from 50 to 59 per cent in 251 animals or 42.2 per cent.

Compared with healthy stock, it will be seen that a great number of animals belonging to leucemic strains contained a very high relative number of lymphocytes in the peripheral blood. As a rule, the lymphocytes did not exceed 60 per cent of the blood leucocytes. The survey showed that 57.8 per cent of the animals of leucemic stock had a relative number of lymphocytes higher than 60 per cent, and that in 30 per cent of the animals, the relative number of lymphocytes exceeded 70 per cent, and also that the relative number of lymphocytes in 10.2 per cent of the animals was higher than 80 per cent. Even if a lymphatic blood picture was characteristic of cattle, a relative number of 70 per cent lymphocytes is so high that it must be considered pathologic. Here it is necessary to note that even if relative lymphocytosis and total leucocytosis have been found in equal numbers of animals, it is not the same animals which show these anomalies. However, it is impossible, from the occurrence of a relative lymphocytosis, to draw conclusions on pathologic disturbances, as the causes of relative lymphocytosis are numerous. It is significant, though, that 60 of the animals (17.3%) have shown, in repeated examinations at intervals of half a year, more than 60 per cent lymphocytes in the peripheral blood. However, only 3, or 5 per cent, of these animals were later attacked by clinically manifest leucemia.

Pathologic mononuclear cells were frequently found in the blood of cattle belonging to leucemic strains. Juvenile pathologic mononuclear cells seldom were found, occurring mainly in a single animal. It has been impossible to establish any connection between the occurrence of pathologic mononuclear cells and a later manifest leucemia. Similar cells have never been noticed in the blood of cows originating from leucemia-free stock.

Pathologic mononuclear cells never occurred in 148 animals or 24.8 per cent; occurred in 1 to 4 per cent of 230 animals or 38.6 per cent; in 5 to 9 per cent of 111 animals or 18.8 per cent; in 10 to 14 per cent of 66 animals or 11.1 per cent; in 15 to 19 per cent of 28 animals or 4.6 per cent; and in more than 20 per cent of 13 animals or 2.1 per cent.

The investigations show that pathologic mononuclear cells have been found in 75.2 per cent of the animals belonging to leucemic strains. Even if the number of these cells is small (1 to 4%) in 38.6 per cent of the animals, more than 5 per cent has been found in 36.6 per cent of the animals, and in 17.8 per cent of the animals, they have amounted to more than 10 per cent of the leucocytes. In these cases, the number of pathologic mononuclear cells is so high that it must be an expression of a change in the health of the animals. However, it is impossible to draw from the results of the investigations any conclusion in respect to the factors conducive to the appearance of these cells in the peripheral blood. Pathologic mononuclear cells have constantly been found in 61.5 per cent of cows examined more than once. It is very significant that higher values of these cells—more than 10 per cent—appeared in 6 of the 7 cows which later showed manifest leucemia, *i.e.*, they occurred in the blood of these animals as long as one and a half to two years before clinical evidence of leucemia could be seen.

Among the leucocytes, mononuclear cells amounted to more than 90 per cent in 26 animals or 4.5 per cent; from 80 to 89 per cent in 120 animals or 20.2 per cent; from 70 to 79 per cent in 173 animals or 29.1 per cent; from 60 to 69 per cent in 138 animals or 23.1 per cent; and from 50 to 59 per cent in 139 animals or 23.1 per cent.

In healthy animals, the highest relative number of mononuclear cells did not exceed 70 per cent of the leucocytes. Among animals from stock with constant leucemia, 53.8 per cent of the animals had more than 70 per cent mononuclear cells in the blood, and in 24.7 per cent there was found such an enormous number of mononuclear cells that they exceeded 80 per cent of the leucocytes. Further, it is worth noting that 63.4 per cent of the animals examined more than once had 70 per cent mononuclear cells in the peripheral blood at each

examination. Again it is of special interest to notice that all the animals which later showed manifest leucemia constantly had more than 70 per cent mononuclear cells in the peripheral blood, even when the examination was carried out two years before the disease could be diagnosed clinically.

The investigations indicate that even if animals of leucemic strains do not clinically show leucemic symptoms, they are, in hematologic respect, suffering with a relative mononucleosis, which is due partly to a relative lymphocytosis, partly to the occurrence of a mononuclear cell type never occurring in the blood of healthy animals. However, it must be pointed out that it is incorrect to draw conclusions on the occurrence of a latent leucemia in these animals from these observations as this mononucleosis can be due to different causal factors not related to leucemia. Only a small number of these animals showed clinical leucemia, even after years of observation. Too, the mononucleosis had been a periodic phenomenon in more animals, followed in later examinations by a normal blood picture. In case where mononucleosis was found in repeated examinations without the possibility of stating what was the clinical causal factor, the hematologic investigation did not disclose the cause of the mononucleosis. But as the mononucleosis seems, in these animals, to be permanent, and as the usual causes of such changes in the blood picture can be excluded, it is possible that the changed blood picture expresses a constitutional, hereditary property—a property characterized by a peculiar lability or a peculiar reactivity of the hematologic tissues in the way that the mother cells of the lymphocytes and the reticulo-endothelial cells do react with an extraordinarily abundant formation of new cells in connection with unknown stimuli.

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Length of Gestation in Range Sheep

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IN INVESTIGATING factors concerned in reproduction of range sheep, it became necessary to keep breeding dates on a considerable number of Rambouillet ewes over a two-year period. This made data available on the normal variation in the length of gestation period of range ewes and made it possible to determine the relation of length of gestation to various other factors. These data have been supplemented with previous data with which breeding dates were kept as a part of the breeding program. It was felt desirable to investigate the influence of environmental and hereditary factors on the length of gestation and, further, to determine if variation in length of gestation might be associated with variation in viability of the offspring.

The length of gestation of domestic sheep has been reported by numerous investigators to vary from 138 to 157 days. These reports are summarized in table 1. The breed averages have varied from about 144 to 152 days. Medium wool, mutton breeds such as the Southdown, Hampshire, Shropshire, and Dorset Horn have short gestation periods, their means varying from 144 to 148 days; while fine wool breeds, such as the Merino and Rambouillet, have long periods, varying from 148 to 152 days. Long wool, mutton breeds such as the Lincoln and Romney have gestation periods intermediate in duration, varying from 146 to 149 days. Reports on other breeds indicate a gestation length of about 149 days for Navajo sheep and 151 days for Karakuls. The crossing of breeds usually resulted in gestation periods intermediate in length to those of the two breeds. Individual gestation periods within breeds varied up to fifteen days, while breed averages varied over a range of eight days. Lush[1] reported a standard deviation of two and four tenths (2.4) days on data from various breeds of sheep.

Information on other factors affecting the length of gestation in sheep is rather meager except for the effect of sex of the lamb, which has been shown to have no effect on gestation length (Carlyle and McConnell[2]; Quinlan *et al.*[3]; Bonfert[4]; Bonsma[5]; Kelley[6]). Dry[7] showed longer gestation periods from single births, while Kelley[6] found no significant difference between twin and single gestations. Bonsma[5] found no significant differences in gestation lengths between first and later pregnancies, while Daley and Eastoe[8] found slightly shorter gestation periods from 2- to 4-year-old ewes than from older ewes. Baronov[9] reported a relation between length of embryonic growth and hair growth. Carlyle and McConnell[2] found that lambs born after long or short gestation periods were lacking in strength and vitality.

MATERIAL AND METHODS

Data are presented on 2,499 gestation periods observed from 1922 to 1939 at the United States Sheep Experiment Station and Western Sheep Breeding Laboratory, Dubois, Idaho, from Rambouillet, Corriedale, Columbia, and Targhee range sheep. These sheep were maintained under strictly range conditions typical of the Intermountain West.

The breeding period was usually of about thirty days' duration during the time from October to December. Previous to 1937, data on length of gestation were available on only small numbers of ewes for which breeding dates were kept. In those cases, ewes in heat were determined by a teaser ram at least once daily and either were hand bred or turned with the ram while in heat. In 1937 and 1938, the ewes were bred in pens and each ram was painted on the brisket daily. Ewes bred (marked by ram) were recorded

From United States Sheep Experiment Station and Western Sheep Breeding Laboratory, United States Department of Agriculture, Dubois, Idaho.

once daily. Breeding weights of the ewes were taken just prior to the breeding period.

Lambing took place in sheds during March and April and on the range in May. During most of the lambing, an attendant was on duty throughout the twenty-four hours of each day to care for the ewes and lambs. Lambs were identified, weighed, and other records taken within a few hours never more than twelve hours following birth. Birth dates of lambs born at night were recorded as of the following day. Milk production of the ewes was roughly estimated soon after birth according to the fullness of the udder and the fill of the lamb.

The length of gestation was calculated from the day of breeding to the day of lambing. All data from questionable breeding or lambing dates, or from gestation periods resulting in obvious premature births, were eliminated from the study.

Data on 1,584 gestation periods from Rambouillets observed in 1937 and 1938 were analyzed to determine the effects of age, weight, breeding date, milk production, and type of birth on gestation length. The method of statistical analysis was similar to that suggested by Yates[10] for the analysis of multiple classifications with unequal subclass numbers, usually designated as the method of fitting constants. The method, described by Hazel[11], is designed to eliminate inequalities due to the various classifications by the least squares procedure to obtain unbiased estimates of differences due to year and type of birth and of the regression coefficients on age, weight, breeding date, and milk production.

RESULTS

Rambouillets had the longest gestation periods of the four breeds, averaging 151.4 days for 2,206 gestation periods. Corriedales averaged 149.6 days for 192 periods and Targhees averaged 149.4 days for 23 gestation periods. Columbias had the shortest gestation periods of the breeds studied, with an average of 148.4 days for 78 gestations. Differences in the breed means were significant except for that between the Corriedales and Targhees. Normal gestation periods for all four breeds

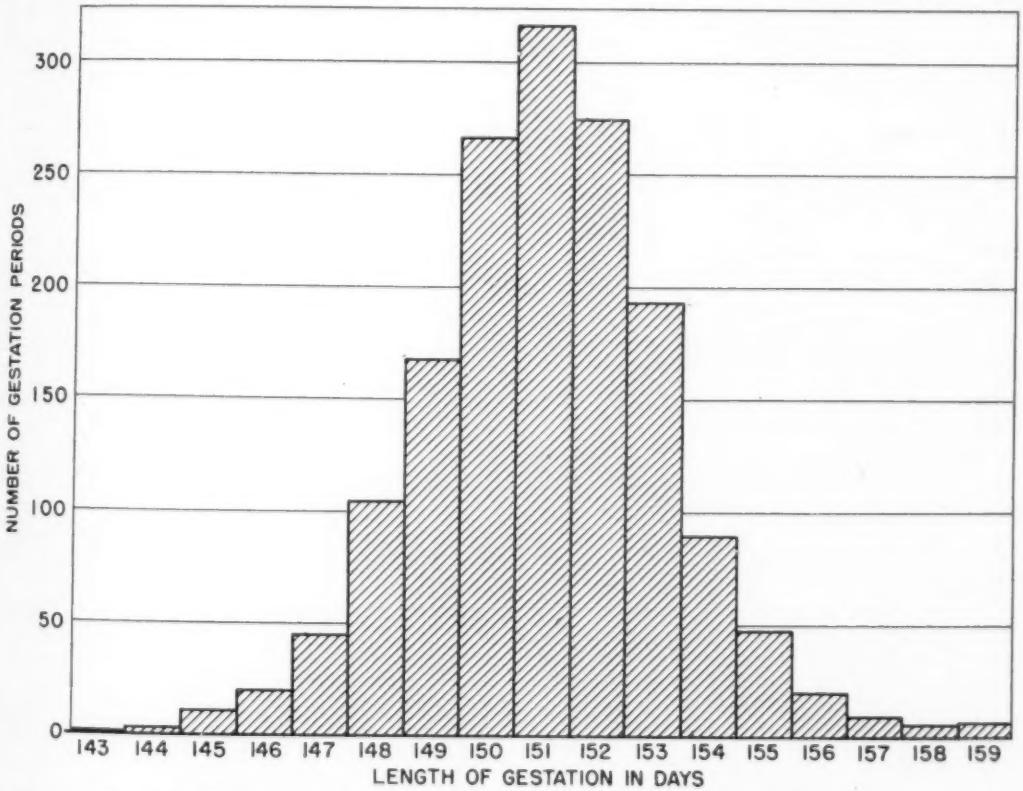

Fig. 1—Frequency graph showing the length of 1,584 gestations of Rambouillet sheep.

varied from 141 to 159 days. Breed differences are sufficiently large that, in predicting the onset of lambing, it is important to consider the appropriate breed average, if available, rather than the species average.

The distribution of 1,584 gestation periods for Rambouillet ewes in 1937 and 1938 are shown in figure 1. These gestations ranged from 143 to 159 days with a mean of 151 days and a standard deviation of two and twenty-three hundredths (2.23) days. The mode was identical with the mean, and the frequency curve appeared to be somewhat flatter than normal.

A summary of analysis of variance for the 1,584 gestation periods, with tests of significance for the different sources of variation, is presented in table 2. The difference between years of eleven hundredths (0.11) day in length of period was not significant.

TABLE 1—Data Reported in Literature on Length of Gestation in Sheep

Average length of gestation (days)	Breed	Range of gestation (days)	No. of gestations	Region	Reference
143.7	Southdown	141 to 148	14†	Missouri	McKenzie & Phillips[11]
144.1	Dorset Horn	138 to 149	101†	Australia	Daley & Eastoe[3]
147.6	Dorset Horn		9	Australia	Kelley[6]
144.6	Hampshire	140 to 147	19†	Missouri	McKenzie & Phillips[11]
144.6	Shropshire	141 to 147	18†	Missouri	McKenzie & Phillips[11]
146.4	Border Leicester X Dorset Horn		10	Australia	Kelley[6]
146.3	Romney			Russia	Elpatjevskii[14]
147.6	Romney			Russia	Vasin[15]
148.7	Romney	144 to 153	107	New Zealand	Dry[7]
148.7	Romney	145 to 153	52	New Zealand	Gill[10]
147.5	Lincoln			Russia	Elpatjevskii[14]
147.9	Border Leicester X Merino		14‡	South Africa	Bonsma[5]
148.1	Heath			Germany	Frolich[17]
148.1	Southdown X Merino		14‡	South Africa	Bonsma[5]
148.3	Dorset Horn X Merino		38‡	South Africa	Bonsma[5]
148.6	Hampshire X Rambouillet	143 to 156	672	Montana	Chittenden & Walker[2]
148.8	Romney X Merino		32‡	South Africa	Bonsma[5]
148.4	Tigai			Russia	Gubanov[13]
149.0	Tigai	141 to 154	147†	Roumania	Bonfert[4]
149.0	Merino X Dorset Horn		32	Australia	Kelley[6]
149.4	Navajo		2130	New Mexico	Blunn[1]
149.5	Precoce			Russia	Vasin[15]
149.5	Valahian			Russia	Vasin[15]
149.6	Hissar			Russia	Vasin[15]
149.6	Ronderib-Africaner	145 to 155	41†	South Africa	Quinlan *et al.*[20]
149.7	Valahian X Karakul			Russia	Vasin[21]
150.3	Rveland X Merino		45‡	South Africa	Bonsma[5]
148.4	Rambouilett (grade)*	143 to 154	50	Oklahoma	Darlow & Hawkins[22]
150.5	Rambouillet	143 to 157	800	Montana	Chittenden & Walker[2]
151.2	Khorasen			Russia	Gubanov[13]
148.5	Merino			Germany	Frolich[17]
149.0	Merino	142 to 156	338†	South Africa	Quinlan & Mare[23]
150.5	Merino		39‡	South Africa	Bonsma[5]
150.9	Merino	146 to 156	109	South Africa	Quinlan *et al.*[3]
151.3	Merino		91	Australia	Kelley[6]
150.8	Karakul			Russia	Baranov[9]
151.4	Karakul			Russia	Vasin[15]
151.4	Karakul			Russia	Baranov[9]
151.7	Karakul			Russia	Gubanov[13]
151.8	Karakul	140 to 155		Russia	Petrov[24]

*Breed of sire was not given.
†Number of ewes.
‡Number of lambs.
Superior figures in last column are references cited.

Age of ewe was the most important environmental factor which affected length of gestation, accounting for 2.8 per cent of the total variance. Ewes varied in age from 2 to 9 years. There was an average increase of twenty-seven hundredths (0.27) day for each advancing year of age. The effect of weight of ewe at breeding time on length of gestation, though positive, was too small to be significant.

Ewes that were bred early in the season tended to have longer gestation periods than ewes that were bred late. The average decrease of three hundredths (0.03) day in length of gestation with the progress of each day in the breeding period was highly significant. However, date of breeding was not an important source of variation, since it accounted for only 1 per cent of the total variance. A curvilinear regression for date of breeding fitted the data only slightly better than a linear regression, despite an apparent slight increase in length of gestation during the last few days of the breeding season. The seeming curvilinearity may have been due to the relatively few pregnancies occurring during the latter part of the period.

There was a significant, but not important, relationship between the rating of milk production of the ewe at the time of birth and the duration of the gestation period. Ewes rated as having good milk production had gestations which averaged forty-five hundredths (0.45) day longer than those rated as poor. It appears likely that slightly increased milk production is a consequence of longer gestation periods.

Gestation periods in which single lambs were born averaged six tenths (0.6) day longer than those in which twin lambs were born. This difference was highly significant, but it accounted for slightly less than 1 per cent of the total variance. The average gestation period for ram lambs was identical with that for ewe lambs. All of the environmental factors studied accounted for about 7 per cent of the total variance.

It was thought that hereditary factors might play an important rôle in determining length of gestation within breeds, since such pronounced breed differences as have been observed are apparently due to hereditary factors also. Gestation periods were sorted according to the sires used, adjusted for the factors mentioned above, and the variance between sires calculated (table 2). It is evident from comparing the variance between sires with the variance within sires that there is considerably more similarity in the gestation periods of ewes mated to the same sires than would have been expected by chance alone. It seems logical that a large part of the similarity was caused by the common hereditary factors which each sire transmitted

TABLE 2—Analysis of Variance for Length of Gestation

Source of variation	Degrees of freedom	Sums of squares	Mean square	F(ratio)
Total	1,583	7,897		
Main classes	6	549.39		
Years	1	9.84	9.84	2.11
Type of birth	1	71.50	71.50	15.35*
Age of ewe	1	224.98	224.98	48.29*
Weight of ewe	1	6.63	6.63	1.42
Date of breeding	1	80.54	80.54	17.29*
Milk production of ewe	1	23.75	23.75	5.10*
Within main classes	1,577	7,347.61	4.659	
Between sires	72	1,057.61	14.689	3.51*
Within sires	1,505	6,290.00	4.179	
Between dams within sires	329	1,817.80	5.525	1.45*
Remainder	1,176	4,472.20	3.803	

*Signifies probability of chance occurrence < .01.

to his several offspring. The intraclass correlation between gestation periods of the half-sibs is eleven hundredths (0.11)*. This indicates that hereditary factors have much more influence on length of gestation than any of the environmental factors studied.

Some of the factors which determine the length of gestation must be inherent in the lamb to be born, since the sire influences the length of gestation of the ewes to which he is mated and this influence must be exerted through the lamb. An equal hereditary effect of the mother through the lamb would be expected. If, in addition to these hereditary effects which are exerted through the lamb, there are additional factors (either hereditary or nonhereditary) which are inherent in the ewe which affect the length of her gestation, it is to be expected that the similarity of two or more gestation periods by the same ewe would be even greater than the similarity of several gestation periods from the same sire. This question was investigated by calculating the variance between 402 dams which had gestations in the two years, after first adjusting for the environmental factors measured and for differences between sires. The intraclass correlation between maternal half-sibs was 0.18, which is somewhat larger than the 0.11 for paternal half-sibs and indicates that individual differences in ewes affect the length of gestation in addition to the hereditary effects exerted through the lamb. It seems that at least 40 to 50 per cent of the variance in length of gestation could be attributed to the total of these effects.

The evidence given here indicates that length of gestation is strongly influenced by the heredity of the lamb and by individual physiologic differences between ewes which may or may not have an hereditary basis but are permanent over at least two gestations. This interpretation is substantiated by relatively large breed differences in average length of gestation, and by the fact that the average gestation period for Rambouillet ewes crossed to Hampshire rams was nearer the Rambouillet than the Hampshire average (Chittenden and Walker[12]).

Lambs which were recorded as hairy at birth were born after slightly longer gestation periods than those which were not hairy. The mean difference of five tenths (0.5) day was barely significant. Only a small proportion of the total lambs born were included in the hairy group.

A significant relationship was found between the length of gestation and viability of the lambs as measured by the ratio of lambs weaned to lambs born. With each day of increase in the length of gestation, there was an average increase of 1.3 per cent of single lambs weaned and 0.6 per cent of twin lambs weaned. The relationship was not strictly linear as there was a tendency for the curves to level off after 152 days for twin lambs and 154 days for single lambs. In general, gestation periods slightly above average in length were most favorable for survival of the lamb.

SUMMARY

The length of 2,499 gestation periods of range sheep have been determined at the United States Sheep Experiment Station and Western Sheep Breeding Laboratory, Dubois, Idaho. Normal periods ranged in length from 141 to 159 days. Rambouillets had the longest gestation periods, averaging 151.4 days, and Columbias had the shortest with an average of 148.4 days. There was little difference between Corriedales and Targhees with averages around 149.5 days.

An analysis of factors affecting the length of gestation was made from 1,584 gestations from Rambouillet ewes obtained in two years. These periods ranged from 143 to 159 days in length with a mean of

*Many of the half-sibs were produced in inbred lines from sires and dams that were related so they were more closely related than the 0.25 for half-sibs in non-inbred populations. After adjusting for the effects of inbreeding, it appeared that between 30 and 40 per cent of the variance in length of gestation period could be attributed to hereditary factors which had direct effects upon the lambs.

151 days and a standard deviation of two and twenty-three hundredths (2.23) days.

Age of ewe was the most important nonhereditary source of variation in length of gestation. There was an average increase of twenty-seven hundredths (0.27) day for each advancing year of age. Weight of ewe at breeding time did not have a significant effect on length of gestation. Ewes bred early in the season tended to have slightly longer gestation periods than ewes that were bred late. Rating of milk production had a slightly positive relationship with gestation length. Gestation periods in which single lambs were born averaged six tenths (0.6) day longer than those in which twin lambs were born. Sex of the lamb had no effect upon length of gestation. All of the environmental factors studied accounted for about 7 per cent of the total variance.

The intraclass correlation between the gestation periods for lambs which were paternal half-sibs was eleven hundredths (0.11), as compared with eighteen hundredths (0.18) for lambs which were maternal half-sibs. This indicates that the heredity of the lambs exerts a strong influence upon length of gestation, augmented further by individual differences between ewes which are evident even when they are mated to different sires in successive seasons. While no effort was made to separate the hereditary effects as exerted through the lamb from those effects inherent in the ewe (either hereditary or nonhereditary), it seemed that at least 40 to 50 per cent of the variance in length of gestation could be attributed to these hereditary factors. This indicates that gestation length could be changed fairly rapidly by selection if that were considered desirable.

Hairy lambs were born after slightly longer gestation periods that nonhairy ones. Gestation periods slightly longer than average favored survival of the lambs after birth.

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Research Fellowships Available

The Research Council of the American Veterinary Medical Association announces that a number of veterinary research fellowships are available for the academic year 1947-48.

Holders of fellowships must be veterinarians and must be citizens of the United States or Canada. Veterinary students who expect to graduate at the end of the current scrool year may apply for fellowships for the following year. The last date for filing applications for the year 1947-48 is March 1, 1947.

The Committee on Fellowships of the Research Council will meet early in April to consider applications and to award fellowships. Applicants will be notified of the action on their applications not later than May 15, in most cases earlier.

The stipend of the fellowships will be determined by the needs of the different fellows and usually will range from $100 to $150 per month.

For information concerning the fellowship project of the Research Council, refer to the February, 1946 (p. 107), issue of the JOURNAL of the AVMA.

It is planned to publish last a list of suggested reesearch projects for the general information of applicants.

For application blanks and other information write:

Dr. H. H. Dukes, *Secretary*
A. V. M. A. Research Council
New York State Veterinary College
Cornell University
Ithaca, New York

Studies on Baby Pig Mortality
IV. Chemistry of the Blood During Fasting and Refeeding of Weanling Pigs

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IN 1941, Graham, Sampson, and Hester[1] described a fatal disease of swine which they called "baby pig disease" or "acute hypoglycemia" in newborn pigs. The disease was designated by these names because it had been observed primarily in pigs 3 to 5 days old and because analysis of the blood of affected pigs invariably indicated an intense hypoglycemia, especially during the late stage of the disease. Subsequently, Sampson, Hester, and Graham[10] reported that a syndrome indistinguishable from the natural disease could be produced experimentally by fasting healthy, newborn pigs for as short a time as thirty-six to forty-eight hours. Intense hypoglycemia was present during the late stage of the experimentally induced syndrome. Experimental evidence suggested that hypoglycemia is a significant part of the physiopathology of both the naturally acquired and experimentally induced syndromes inasmuch as improvement and sometimes recovery of affected pigs followed repeated glucose injections. Results of further studies in this laboratory appear to indicate that severe hypoglycemia was the primary, significant change in the blood of these pigs.

The foregoing observations, in contrast to the knowledge that prolonged fasting apparently does not cause a dangerous fall in blood sugar of well-grown dogs as demonstrated by Morgulis and Edwards[7] in 1924, prompted the authors to study the effect of fasting and refeeding upon the sugar and certain other constituents of blood in weanling pigs.

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Assistant in Animal Pathology and Hygiene (Hanawalt) and professor and chief in Animal Pathology and Hygiene (Sampson).

EXPERIMENTAL

Fasting experiments were carried out with two groups of weanling pigs, one in the late fall of 1943 and the other in the winter of 1945 and 1946. The pigs used in these trials ranged in weight from 20 to 39.5 lb. and were divided into experimental and control groups on the basis of breed, sex, and weight. In trial 1, the average weight of 4 experimental pigs was 35 lb. and that of 4 control pigs, 31 lb. Five experimental pigs used in trial 2 averaged 25.7 lb. and 4 control pigs, 28.3 lb. Both groups of pigs had been hand fed before the experiments started but, in the first trial, the experimental group had access to a self-feeder because it was assumed that under these conditions there would be maximum absorption from the intestinal tract. Results obtained during fasting did not seem to bear out this assumption because the individual blood-glucose concentrations of the nonfasted pigs varied on consecutive bleedings and, therefore, the experimental pigs were hand fed in trial 2. A weighed amount of feed was fed to the control group each morning and the pigs were allowed to eat for approximately two hours. The ration fed in both experiments was a mixture of 600 lb. ground yellow corn, 250 lb. ground oats, 50 lb. tankage, 50 lb. soybean oil meal, 40 lb. alfalfa meal, and 10 lb. mineral mixture consisting of 40 per cent steamed bone meal, 40 per cent limestone, and 20 per cent salt. None of the pigs in either experiment had access to grass. Water was allowed *ad libitum* to all groups. All pigs were kept on concrete floors except the experimental pigs in trial 2, which were placed in a large metabolism crate equipped with a screened floor so all fecal material could be promptly removed. In neither experiment, however, was evidence of coprophagy observed. During the first trial, all pigs were kept in pens in an unheated shed whereas, in trial 2, the pigs were confined in pens in a room where the temperature ranged from 20 to 28 C. (68 F. to 84 F.)

The experimental pigs in trial 1 were subjected to twenty-four days of fasting during which time there was marked loss in body weight. However, the fasted pigs were fairly strong and active at the end of this period. The experimental pigs in trial 2 were fasted twenty-eight days and again the pigs were relatively alert at the end of this interval, although they did not seem to be as strong as the fasted group during the first trial.

Blood was collected over sodium fluoride by means of heart puncture during trial 1 but, in trial 2, all pigs were bled from the anterior vena cava according to the technique described by Carle and Dewhirst[2]. The latter procedure was preferable because, thereby, it was possible to bleed each pig several times in a single day. In the first trial, each pig was bled at approximately five-day intervals whereas, in the second trial, samples of blood were taken every other day except near the beginning and end of the fast when several samples were obtained for analysis on the same day.

At the conclusion of the fast in trial 2, autopsies of 3 of the experimental pigs failed to reveal evidence of significant injury to the tissues from the bleeding technique.

Methods of Chemical Analyses.—The methods employed for blood analyses in trial 1 were as follows: sugar — Shaffer, Hartmann, Somogyi technique according to Koch[6]; inorganic phosphorus — Youngburg[13]; iron and hemoglobin — Wong[11]; creatinine — Folin-Wu[4]; ketone bodies — Van Slyke-Fitz[3]; CO_2 capacity of plasma — Van Slyke[3]. Readings for iron and creatinine were made on a Klett-Summerson photoelectric colorimeter. In the second experiment, only blood sugar and hemoglobin determinations were made and the hemoglobin values were read directly on a Fisher electro-hemometer.

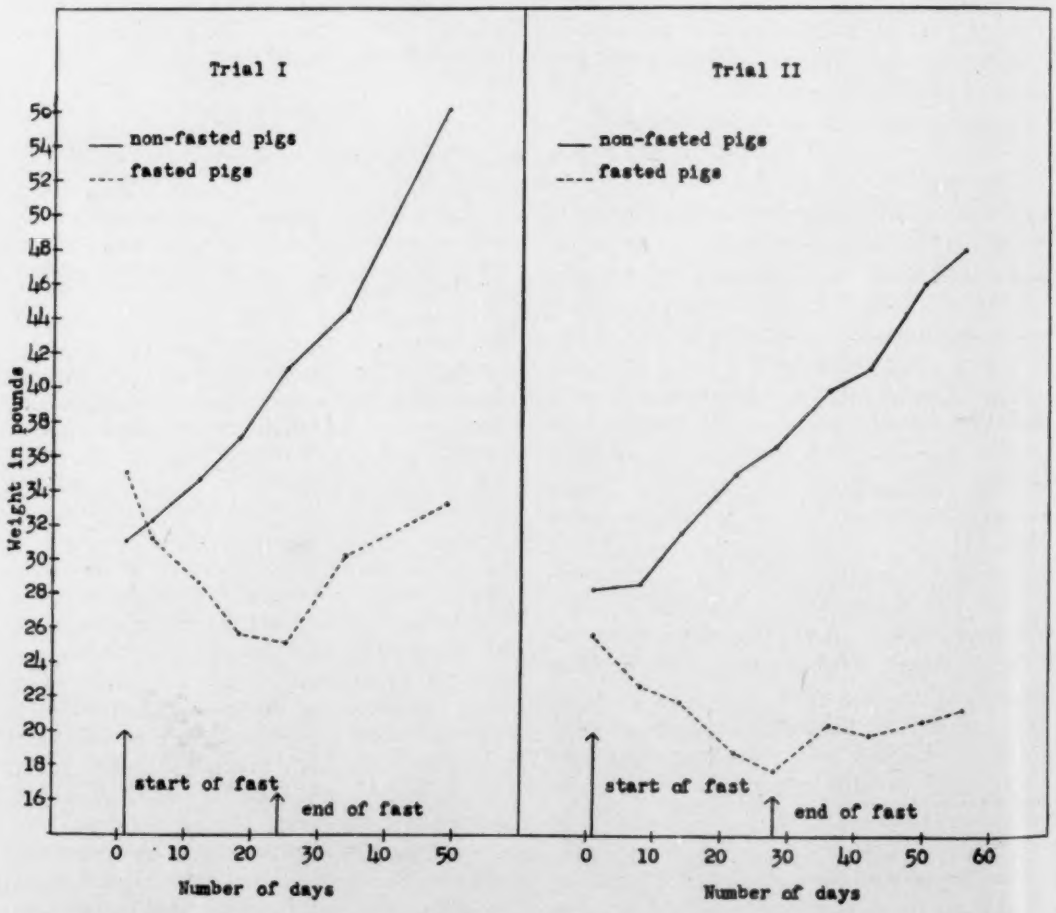

Fig. 1—Body weights during fasting and refeeding of weanling pigs. The values represent averages for each group.

TABLE I—Effect of Fasting and Refeeding on the Blood Glucose Concentration of Weanling Pigs in Trial 2

Time (days)	Nonfasted pigs (controls)					Fasted pigs					
	Pig no. R_3	R_4	L_1R_2	L_3R_3	Av.	Pig no. R_1	R_2	R_5	L_1R_3	L_2R_3	Av.
Fasting Period (28 days)											
1 (a)	85.88	79.10	79.10	74.58	79.7	90.40	70.26	70.26	83.62	65.44	76.0
(b)	83.62	72.32	79.10	65.44	75.1	79.10	76.84	64.31	83.62	70.26	74.8
(c)	65.44	67.80	67.80	63.18	66.1	67.80	67.80	63.18	76.84	59.32	67.0
(d)	83.62	67.80	67.80	65.44	71.2	81.36	67.80	54.24	81.36	70.26	71.0
2 (a)	70.26	54.24	54.24	54.24	58.2	70.26	79.10	56.50	54.24	38.42	59.7
(b)	85.88	83.62	85.88	83.62	84.8	74.58	61.02	47.46	61.02	33.90	55.6
3 (a)	67.80	72.32	67.80	72.32	70.1	45.20	61.02	51.98	54.24	42.94	51.1
(b)	81.36	65.44	83.62	76.84	76.8	51.98	67.80	49.72	63.18	47.46	56.0
4 (a)	79.10	67.80	76.84	67.80	72.9	56.50	58.76	51.89	54.24	51.89	54.7
(b)	74.58	79.10	67.80	70.26	72.9	61.02	58.76	49.72	56.50	51.98	55.6
5	65.44	74.58	65.44	67.80	68.3	61.02	49.72	49.72	51.98	51.98	52.9
7	70.26	74.58	72.32	65.44	70.7	58.76	49.72	45.20	49.72	58.76	52.4
9	64.97	73.45	36.72	58.76	58.5	67.80	47.46	45.20	51.98	47.46	52.0
11	70.26	81.36	72.32	63.18	71.8	61.02	40.68	47.68	33.90	58.76	48.4
13	74.58	72.32	65.44	61.02	68.3	67.80	51.98	54.24	56.50	65.44	59.2
15	63.18	76.84	70.26	63.18	68.4	63.18	47.46	45.20	58.76	49.72	52.9
17	70.26	72.32	70.26	63.18	69.0	61.02	38.42	38.42	56.50	31.64	45.2
19	67.80	74.58	63.18	65.44	67.8	70.26	45.20	51.98	58.76	49.72	55.2
21	72.32	74.58	56.50	63.18	59.1	65.44	45.20	40.68	58.76	45.20	51.1
23	65.44	67.80	67.80	63.18	66.1	57.86	42.94	42.94	54.24	45.20	48.6
25	72.32	67.80	61.02	61.02	65.5	63.18	42.94	51.98	51.98	38.42	49.7
27	67.80	67.80	54.24	67.80	64.4	67.80	40.68	38.42	47.46	36.16	46.1
29 (a)	63.18	70.26	63.18	65.44	65.4	67.80	47.46	51.98	63.18	58.76	57.8
Refeeding Period (28 days)											
29 (b)	76.84	99.44	103.96	117.52	99.4	185.30	108.48	155.94	115.26	171.70	147.3
(c)	81.36	83.62	83.62	92.66	85.3	223.75	131.08	221.48	162.72	216.96	191.2
(d)	70.26	99.44	56.50	74.58	75.2	205.66	76.84	198.88	99.44	185.32	153.2
30 (a)	67.80	79.10	58.76	63.18	67.2	70.26	63.18	88.14	63.18	115.26	80.0
(b)	74.58	83.62	72.32	70.26	75.2	76.84	54.24	94.92	70.26	119.78	83.2
31	74.58	65.44	61.02	63.18	66.1	79.10	58.76	67.80	70.26	61.02	67.4
33	61.02	67.80	54.24	61.02	61.0	76.84	58.76	79.10	67.80	70.26	70.6
35	67.80	72.32	61.02	58.76	65.0	65.44	67.80	79.10	67.80	65.44	69.1
37	67.80	74.58	63.18	65.44	67.8	83.62	67.80	72.32	70.26	74.58	73.7
39	70.26	81.36	63.18	61.02	69.0	76.84	63.18	76.84	58.76	83.62	71.9
41	63.18	67.80	54.24	56.50	60.4	79.10	67.80	63.18	47.46	79.10	67.3
43	67.80	65.44	61.02	58.76	63.3	61.02	72.32	67.80	63.18	76.84	68.2
45	67.80	74.58	65.44	63.18	67.8	76.84	70.26	70.26	67.80	83.62	73.8
47	61.02	70.26	56.50	70.26	64.5	67.80	61.02	61.02	56.50	65.44	62.4
49	67.80	65.44	63.18	61.02	64.4	65.44	67.80	63.18	56.50	67.80	64.1
51	67.80	76.84	54.24	83.62	70.6	63.18	65.44	67.80	63.18	67.80	65.5
53	65.44	70.26	56.50	56.50	62.2	65.44	63.18	61.02	65.44	63.18	63.7
55	58.76	65.44	54.24	51.98	57.6	70.26	56.50	65.44	63.18	65.44	64.2
57	70.26	76.84	51.98	54.24	63.3	56.50	72.32	65.44	61.02	63.18	63.7

a, b, c, and d designate consecutive bleedings at approximately two-hour intervals on the same day.

RESULTS

During the inanition periods in both experiments, the fasted pigs showed a marked decrease in body weight, whereas the nonfasted pigs, as was to be expected, showed an increase. At the end of the 24-day fast in trial 1, the group deprived of all feed had lost an average of nearly 10 lb. per pig, or approximately 28 per cent of the initial body weight, while the control pigs had gained almost 26 per cent in weight during that time, representing an average increase of 8 lb. per pig. Both the nonfasted and fasted pigs in the second experiment lost appreciable weight the first week after the start of the fast. This was attributed, in part, to the fact that the control animals ate poorly for several days and because possibly they were not adjusted to handling or to the new environment, despite the fact that these pigs were placed in their experimental pens and bled twice during the week prior to the experiment. The pigs fasted twenty-eight days lost an average of about 8 lb. per animal constituting a 30 per cent reduction in body weight. In contrast, the controls gained an average of 8 lb. per pig, representing a 30 per cent increase. The pigs used in the second trial averaged 6.5 lb. less in body weight when the experiment started than the pigs in trial 1, and this may explain why these pigs lost less weight per pig during the fast. However, the total percentage loss in weight was greater for the pigs in the second experiment, a result which might be anticipated, because these pigs were fasted four days longer than those in the first trial (*see* fig. 1).

Following twenty-four days of refeeding, the fasted pigs in trial 1 averaged 33.2

Fig. 2A—Effects of fasting and refeeding on weanling pigs. Nonfasted control group; (a) at beginning of experiment, (b) after twenty-eight days, (c) after fifty-six days, and (d) eighty days after termination of experiment (2 control pigs succumbed to hog cholera).

lb. and the control pigs 55.5 lb. It was interesting to note that after twenty-four days of refeeding the average weight of the fasted pigs was 2.4 lb. greater than the average of their original weights; thus, the fasted pigs required approximately the same number of days of refeeding as were spent in fasting to regain the weight lost during inanition. The gains of the fasted pigs during refeeding were not as high in trial 2, for these pigs averaged 4.3 lb. less in weight than when the fast began and only 1 pig out of the 5 had completely regained its initial weight. Since these pigs were fasted four days longer than the previous group, it would appear on the basis of the limited numbers that the longer fast caused slower recovery. After twenty-eight days of refeeding, the average weight of the fasted pigs was 21.4 lb. while that of the nonfasted pigs was 48 lb. (*see* fig. 1). Figures 2A and 2B show photographs of the non-fasted and fasted pigs in trial 2 at the beginning of the experiment, near the end of the fast, twenty-eight days after refeeding, and eighty days after the termination of the experiment.

At the end of the 28-day fast, the fasted pigs were placed in a metabolism crate and a 24-hour urine specimen collected from each pig. Qualitative determinations on the urine indicated there were no ketone bodies, albumin, sugar, hemoglobin, or bile salts in any of the samples. Thus, it appears that the 28-day fast in this experiment caused no nephritis or nephrosis.

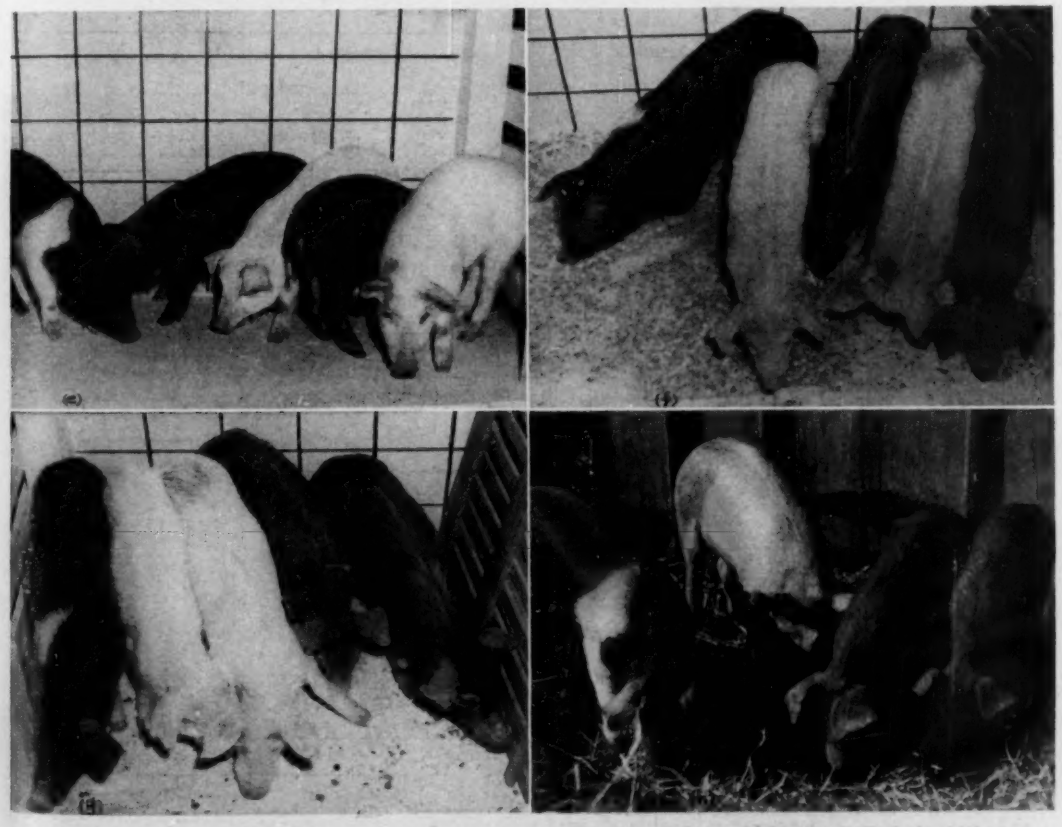

Fig. 2B—Effects of fasting and refeeding on weanling pigs. Fasted group: (e) at beginning of experiment, (f) after twenty-eight days of fasting, (g) after twenty-eight days of refeeding, and (h) eighty days after termination of experiment (1 pig died with pneumonia).

Note evidence of marked loss of body weight and unthriftiness of pigs after twenty-eight days of fasting in contrast to the nonfasted pigs (f), (b). Note improvement in physical condition and recovery of pigs from the effects of fasting (g), (h).

In the second experiment, the body temperature of each pig was recorded at weekly intervals. The average value for the fasted pigs during the period of inanition was 100.7 F., but at the close of the refeeding period it had risen to 102.3 F. The controls, on the other hand, maintained an average temperature of 103.3 F. throughout the entire experiment. This apparent difference in thermo-regulation presents a problem meriting further investigation.

Approximately eighty days after the termination of trial 2, the 6 remaining pigs, 2 controls, and 4 previously fasted pigs were again weighed, temperatures recorded, and blood collected for glucose and hemoglobin determinations. Blood glucose concentrations were normal for both groups, and averaged 56.50 mg. per 100 cc. of blood for the control pigs and 68.34 mg. per 100 cc. for the previously fasted pigs. The average temperature of the control pigs was 103.3 F. and that of the previously fasted pigs 103.7 F. Hemoglobin values for both groups also were in the normal range, averaging 12. 2 Gm. per 100 cc. for the control and 10.3 Gm. per 100 cc. for the previously fasted pigs. Weight gains, on the other hand, were not as consistent. The 2 control pigs averaged 99.8 lb. in weight, representing an average increase of 60 lb. in eighty days or a gain of 3/4 lb. per day. The 4 previously fasted pigs had an average weight of 62.8 lb. and gained an average of 39.5 lb. in eighty days or about 1/2 lb. a day. There was, however, considerable difference in the weight gains of the 4 previously fasted pigs since their individual weights varied from 43.0 to 97.5 lb. One experimental pig, designated as R_1, weighed more than 1 of the controls and had gained at the rate of almost 9/10 lb. per day, indicating a 28-day fast had not produced a permanent injury in this particular pig. It was also interesting to observe that this pig maintained the highest blood sugar level of the experimental pigs during the entire period of inanition and had the highest blood-sugar concentration when the pigs were bled eighty days after the experiment ended. Figure 2B shows that the previously fasted pigs, although small, had a smooth hair coat and appeared to be in a good state of nutrition.

Results of chemical determinations indicated that a fast of twenty-four to

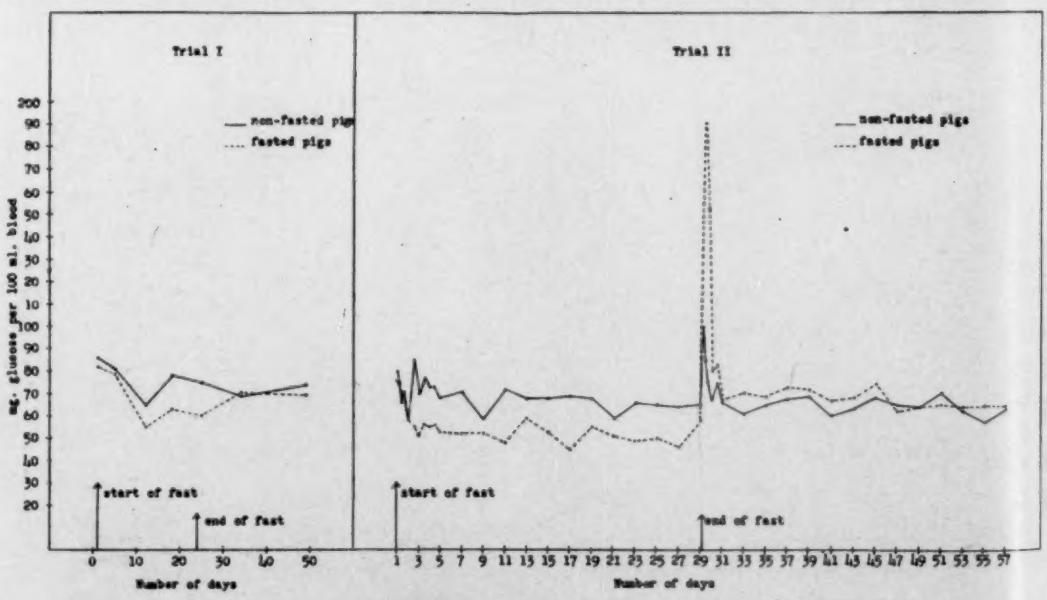

Fig. 3—Blood glucose during fasting and refeeding of weanling pigs. The values represent averages for each group.

twenty-eight days' duration caused no gross alterations in the chemistry of the blood of weanling pigs. Physiologic changes in the concentration of certain constituents, however, seemed to be associated with fasting as follows:

Sugar.—In trial 1, the blood sugar decreased during the early stages of the fast and then increased, but not to the prefast level. This level was not attained until refeeding started. The sugar level of the blood of control pigs showed relatively wide fluctuations throughout, but at no time did it reach as low a value as observed in fasted animals. This variation may be ascribed to the fact that the pigs ate from a self-feeder and thus controlled their own eating schedules (*see* fig. 3, trial 1). This problem was partially eliminated in the second trial by bleeding the control pigs before feeding each morning so that none had consumed food for almost twenty hours preceding the collection of the blood sample except on those particular days when it was necessary to bleed more than once. Data in table 1 for trial 2 show that the blood-sugar levels of the fasted pigs decreased significantly at the beginning of the fast and the low range was maintained throughout the remainder of the period of inanition, whereas values for control pigs remained at a higher level over the same interval; nor did the values for either the control or fasted pigs vary markedly during the twenty-eight days. These observations on the effect of fasting on blood sugar in weanling pigs are in general accord with results reported by Morgulis and Edwards' for blood sugar in the fasting dog.

On the morning of the twenty-ninth day, all fasted pigs were put back on the regular ration shortly after bleeding and each pig consumed about 1/2 lb. of feed. Each pig in the control group was eating approximately 3 lb. of feed per day at this time. Although the fasted pigs were weak and inactive, they were hungry and ate readily just as soon as the food was placed in the pen. Both the fasted and nonfasted pigs were bled four times on this particular day at approximately two-hour intervals. The values obtained for the blood sugar of the fasted pigs appeared to have considerable significance. The average blood sugar concentration at the time of the second bleeding, made one hour after the pigs had eaten for the first time in twenty-eight days, was 147.33 mg. per 100 cc. of blood and the average for the controls was 99.44 mg. per 100 cc. The pigs continued to eat for another hour and were then bled

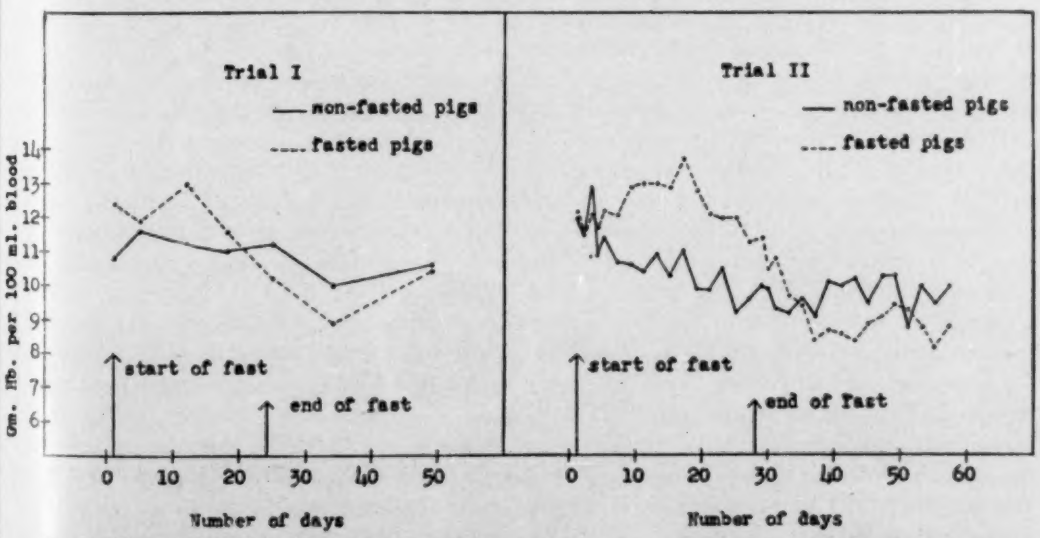

Fig. 4—Hemoglobin during fasting and refeeding of weanling pigs. The values represent averages for each group.

again. This time the average blood sugar concentration for the fasted pigs attained a peak of 191.19 mg. per 100 cc. of blood, whereas the control pigs had an almost normal average concentration of 85.31 mg. of glucose per 100 cc. of blood. The blood-sugar concentration for the 5 fasted pigs remained high throughout the day and, at the time of the fourth bleeding, it was 153.25 mg. while that of the controls was 75.2 mg. per 100 cc. of blood. By the next morning, however, the average value for the blood glucose of the fasted pigs had dropped to a normal level of 75.2 mg. and showed no significant rise during that day even after the pigs had been fed (*see* fig. 3, trial 2 and table 1).

TABLE 2—Data on Blood Glucose, Hemoglobin, Body Weights, and Temperatures of 6 Pigs Studied in Trial 2 Eighty Days after the Termination of the Experiment

	Nonfasted pigs (controls)			Previously fasted pigs		
	Pig No. L_1R_2	L_2R_3	Pig No. R_1	R_3	L_1R_3	L_2R_2
Blood glucose (mg. per 100 cc. blood)	61.02	51.98	76.84	72.32	58.76	65.44
Hemoglobin (Gm. per 100 cc. blood)	12.9	11.4	10.7	10.6	9.3	9.7
Weight of pigs (lb.)	105.6	94.0	97.5	47.0	63.5	43.0
Temperature of pigs (Fahrenheit)	103.7	102.8	104.1	103.4	103.4	103.7

In this connection, it would appear that following a period of inanition, low carbohydrate intake, and undernutrition young, growing pigs develop some impairment of carbohydrate metabolism. Russell[9] has specified that this impairment of metabolic activity involves the liver and possibly the peripheral tissues and that it might be due to a lack of, or delay in, insulin secretion. It should be borne in mind that a period of adaptation may be required by certain organs other than the liver before normal carbohydrate metabolism is resumed. It is known that endocrine glands other than the pancreas have an influence upon carbohydrate metabolism as the adrenal, pituitary, and thyroid glands all are associated with this function. According to Haist[5], two conditions may reduce the amount of insulin: (1) that which cuts down the demand such as fasting, low carbohydrate-high fat diets, and the administration of insulin; and (2) that which increases the demand for insulin including partial pancreatectomy or treatment with anterior pituitary extracts. Apparently, a 28-day fast in weanling pigs results in only a temporary impairment of carbohydrate tolerance because within twenty-four hours after refeeding the blood-glucose concentrations of the experimental pigs were at the same level as those of the controls. This observation seems to vary somewhat from results obtained for the rat. For example, Best and Taylor[1] state that the insulin content of the pancreas of rats starved or fed a diet rich in fat for seven days decreased to about half the normal value and was not replenished until six days after the rats were returned to a balanced diet.

Inorganic Phosphorus.—There was some evidence in trial 1 that fasting resulted in a slight decrease in the inorganic phosphorus content of blood, but results were inconclusive.

Hemoglobin.—Apparently, fasting caused no significant alterations in hemoglobin values although in both experiments average values obtained at the end of fasting were lower than the average for prefasting determinations. Even though the fasted pigs had constant access to water, it appears they might have been dehydrated, resulting in some hemoconcentration which

may partly explain the rise in hemoglobin values in both groups during fasting. Shortly after refeeding, the concentration of hemoglobin for the previously fasted pigs decreased to a level near that of the control pigs and apparently remained at this level throughout the refeeding interval (*see* fig. 4).

CO_2 Capacity of Plasma.—In trial 1, there was an appreciable rise in the CO_2 combining power of the blood during the late stage of inanition. In the control animals, however, it remained relatively constant. The significance of the increase in the CO_2 capacity of the blood of fasting pigs is not clear. No appreciable concentration of ketones appeared in the blood during inanition, but an increase in alkali reserve would hardly be expected in the fasting state. Further study of the effect of fasting on acid-base balance in pigs may provide an explanation.

Total Ketone Bodies.—Only a trace of ketone bodies was found in the blood of fasting pigs and none was observed in the blood of control pigs.

Creatinine.—Fasting gave rise to no significant change in creatinine content of the blood of weanling pigs. Morgulis and Edwards[7] made a similar observation on blood creatinine in dogs.

SUMMARY

This report presents comparative data obtained in chemical determinations on (1) the blood of two groups of weanling pigs fasted for twenty-four and twenty-eight days, respectively, and then refed the same number of days, and (2) the blood of two groups of weanling pigs not fasted and used as controls. Data on body weights and temperatures of fastings pigs are also reported.

Apparently, a fast of twenty-four or twenty-eight days (water, *ad libitum*) produced no pathologic changes in the chemical components of the blood of these pigs for which determinations were made.

During fasting and refeeding, physiologic changes occurred in some chemical constituents. Blood sugar concentrations decreased somewhat during fasting and then increased during refeeding to a concentration approximating that of the controls. Fasting produced no ketonemia. In the late stage of fasting, there was an unexplained rise in CO_2 combining power of the blood. Apparently, inanition gave rise to no significant alterations in the concentration of inorganic phosphorus, hemoglobin, or creatinine of the blood.

There was an appreciable decrease in body temperature of pigs during fasting, even though they were kept in a warm room.

Pigs subjected to a 24-day period of inanition lost approximately 28 per cent of their initial body weight while those fasted twenty-eight days lost 30 per cent of their weight.

Prolonged fasting of weanling pigs apparently gave rise to a temporary impairment of carbohydrate metabolism.

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The Behavior of the Peripheral Blood Elements in Panleucopenia (Agranulocytosis) of the Domestic Cat

WAYNE H. RISER, D.V.M., M.S.

Des Moines, Iowa

THE PUBLICATIONS of Lawrence, Syverton, Shaw, and Smith[1] and of Hammon and Enders[2, 3] which reported definite decreases in the peripheral blood elements in cats with panleucopenia (agranulocytosis) stimulated this investigation to attempt to correlate the behavior of these blood changes so that they could be used as a diagnostic aid.

Ten healthy, well-nourished cats, 5 males and 5 females, were used to study panleucopenia by inoculating them with the virus.* The animals were housed, fed, and examined grossly and microscopically.

The symptoms were fairly constant in all of the cases, but there was a marked individual variation in both the course and severity of the condition. All the animals showed only slight signs of illness prior to the crisis period. Weight loss and a rough, unkempt condition of the hair coat were the first symptoms noted. These were seen in every case, even in case 5 which exhibited a very mild reaction. The initial symptoms were followed by vomition in the cases of 2 cats (20%) and diarrhea in the cases of 4 cats (40%). All the animals at the crisis assumed the typical prone position (fig. 1.), and they paid little attention to their surroundings.

In every case, dehydration was evident. It was so extreme in some instances that the skin at the nape of the neck would stand when pulled upward, and it would so remain for as long as one or two minutes.

The ages of the cats in this experiment ranged from 2 to 10 months, the average being 6 months. Death occurred in 8 (80%) of the 10 cases, and all the animals were comatose or in a state of prostration at the time of death.

Fig. 1—A cat showing a typical prone position at terminal stage of panleucopenia (agranulocytosis).

THE BLOOD PICTURE AND TEMPERATURE

The blood picture and temperature changes of panleucopenia were studied in 10 cats inoculated with the virus. An attempt was made to plot the variations in the leucocyte and the erythrocyte counts and the temperature during the course of the disease, with the hope that this information might be of diagnostic value in determining the stage of sickness in cats admitted for treatment.

Observation of the 10 inoculated cases showed that very definite changes took place in the leucocyte and the erythrocyte counts, and the temperature during the course of the disease. One change was probably not more significant than another,

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*I am indebted to Dr. W. S. Gochenour of the Pitman-Moore Co., Indianapolis, Ind., and the Fort Dodge Laboratories, Fort Dodge, Iowa, for supplying the inoculating materials.

Note: The statistical calculations used in this paper were prepared by the Statistical Laboratory at Iowa State College, Ames, Iowa, by methods standard in that field.

TABLE I—Means and Standard Deviation of the Leucocyte Count of the Experimental Clinical Cases

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Day	Cat 1	Cat 2	Cat 3	Cat 4	Cat 5	Cat 6	Cat 7	Cat 8	Cat 9	Cat 10	Standard Deviation	Means
I	15,400	11,500	17,000	16,200	10,800	14,950	17,500	13,050	10,500	15,100	2,569	14,200
1		17,400				10,100	27,000	28,800	18,000	24,100	7,031	20,900
2	11,300	10,200	10,000	21,200	15,000	10,000	6,750	16,000	15,800	16,600	4,321	13,285
3	5,200	6,000	1,350	20,800	8,900	8,200	2,850D	10,200	10,050	12,100	5,477	8,565
4	6,900	4,200	800	18,800	6,500	4,600		5,300	3,600	15,300	5,851	7,333
5	700D	6,800	50D	5,400	2,550	6,200		6,750	4,200	11,700	3,577	4,928
6		2,400		600D	7,500	6,450		3,450	900D	10,800	3,789	4,586
7		350			7,050	2,400		600D		6,250	3,145	3.330
8		300			16,500	425				8,900	7,770	6.531
9		4,300			15,700R	425				10,200	6,701	7,656
10		6,900				13,000				400D	6,301	6,767
11		16,600D				26,700						
12						30,150						
13						28,600R						

I = Day of inoculation.
D = After last number in column means 'Death'.
R = After last number in column means 'Recovery'.

TABLE 2—Means and Standard Deviation of the Erythrocyte Count (in Millions) of the Experimental Clinical Cases

Day	Cat 1	Cat 2	Cat 3	Cat 4	Cat 5	Cat 6	Cat 7	Cat 8	Cat 9	Cat 10	Standard Deviation	Means
I	9.09	8.38	9.22	7.80	6.72	8.36	5.10	7.52	8.04	7.00	1.22491	7.723
1		6.26				7.10	4.40	7.90	6.22	6.72	1.17369	6.433
2	7.08	8.16	7.38	6.99	7.96	7.00	5.50D	8.74	7.06	6.72	.88863	7.259
3	7.20	8.34	7.04	6.84	7.00	7.08		8.34	6.80	6.74	.62680	7.264
4	6.49	8.17	5.56	6.28	6.72	6.90		7.96	6.65	6.10	.83967	6.759
5	9.00D	8.64	6.68D	5.50	6.02	7.12		6.90	6.20	7.20	1.15647	7.029
6		6.27		5.80D	5.66	7.32		6.22	7.06D	7.60	.73381	6.561
7		7.44			5.90	7.92		6.34D		6.76	.81567	6.872
8		8.56			5.60	7.36				7.08	1.21650	7.150
9		6.66			6.26R	6.94				6.50	.28542	6.590
10		4.41				6.10				6.08D	.97000	5.530
11		4.27D				3.34						
12						5.76						
13						7.70R						

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Means

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2	11,300	10,200	10,000	21,200	15,000	10,000	6,750	16,000	15,800	16,600	4,321	13,285
3	5,200	6,000	1,350	20,800	8,900	8,200	2,850D	10,200	10,050	12,100	5,477	8,565
4	6,900	4,200	800	18,800	6,500	4,600		5,300	3,600	15,300	5,851	7,333
5	700D	6,800	50D	5,400	2,550	6,200		6,750	4,200	11,700	3,577	4,928
6		2,400		600D	7,500	6,450		3,450	900D	10,800	3,789	4,586
7		350			7,050	2,400		600D		6,250	3,145	3,330
8		300			16,500	425				8,900	7,770	6,531
9		4,300			15,700R	425				10,200	6,701	7,656
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TABLE 2—Means and Standard Deviation of the Erythrocyte Count (in Millions) of the Experimental Clinical Cases

Day	Cat 1	Cat 2	Cat 3	Cat 4	Cat 5	Cat 6	Cat 7	Cat 8	Cat 9	Cat 10	Standard Deviation	Means
I	9.09	8.38	9.22	7.80	6.72	8.36	5.10	7.52	8.04	7.00	1.22491	7.723
1		6.26				7.10	4.40	7.90	6.22	6.72	1.17369	6.433
2	7.08	8.16	7.38	6.99	7.96	7.00	5.50D	8.74	7.06	6.72	.88863	7.259
3	7.20	8.34	7.04	6.84	7.00	7.08		8.34	6.80	6.74	.62680	7.264
4	6.49	8.17	5.56	6.28	6.72	6.90		7.96	6.65	6.10	.83967	6.759
5	9.00D	8.64	6.68D	5.50	6.02	7.12		6.90	6.20	7.20	1.15647	7.029
6		6.27		5.80D	5.66	7.32		6.22	7.06D	7.60	.73381	6.561
7		7.44			5.90	7.92		6.34D		6.76	.81567	6.872
8		8.56			5.60	7.36				7.08	1.21650	7.150
9		6.66			6.26R	6.94				6.50	.28542	6.590
10		4.41				6.10				6.08D	.97000	5.530
11		4.27D				3.34						
12						5.76						
13						7.70R						

I = Day of inoculation.
D = After last number in column means 'Death'.
R = After last number in column means 'Recovery'.

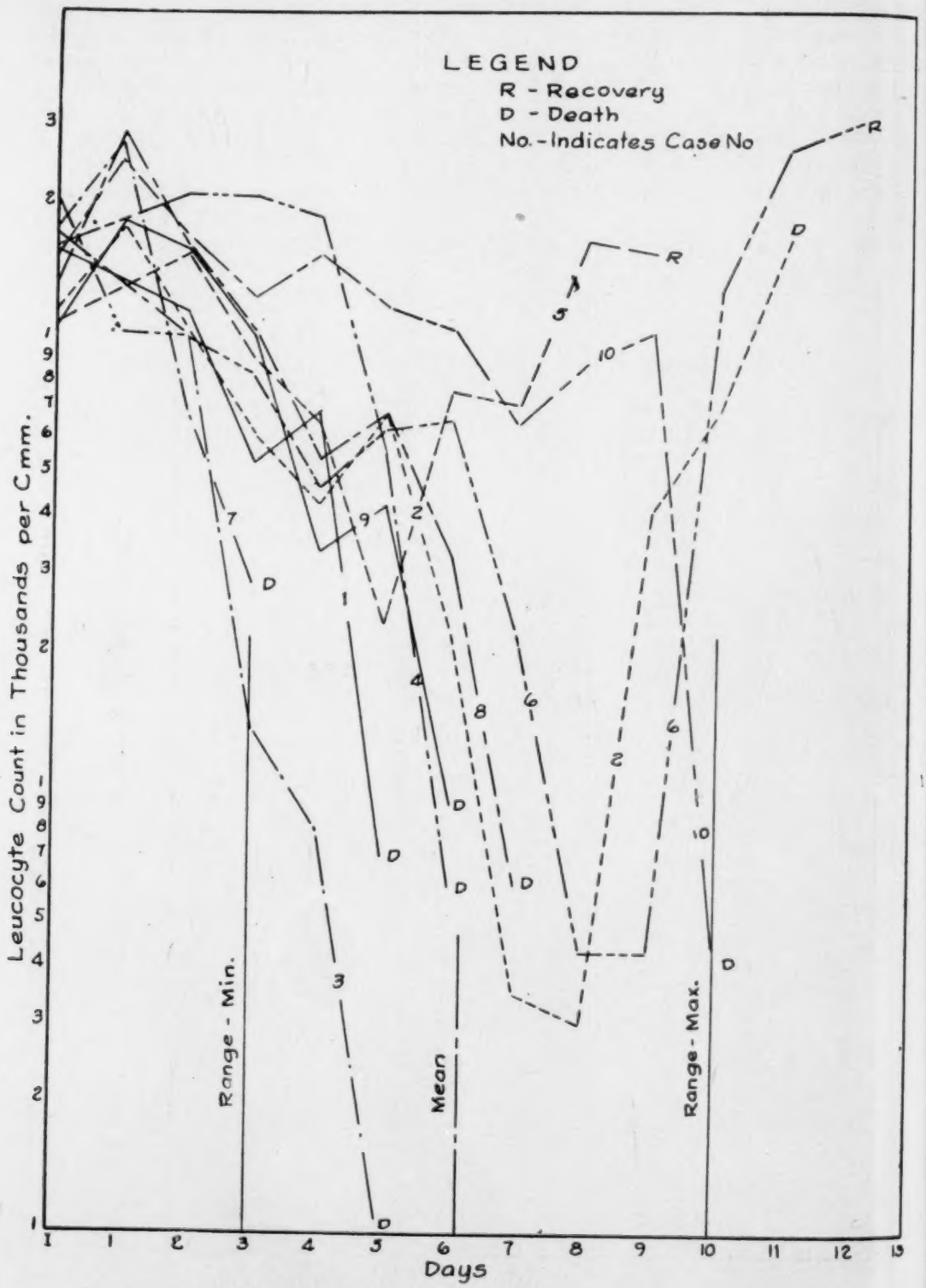

Chart 1—Leucocyte count of the experimental cases showing decrease in cells and crisis days.

but the leucocyte variation was the more easily determined. The leucocytes of the peripheral blood decreased rapidly until they almost disappeared. The lowest leucocyte determination was designated as the crisis. When this was reached, the leucocytes appeared in the blood in increasing numbers as rapidly as they had disappeared.

It was found from this investigation and from the work of others[4, 5] that leucocyte counts rarely fell below 10,000 cells per cubic millimeter in normal adult cats, and 8,000 cells per cubic millimeter in normal kittens under approximately 3 months of age. These counts were so consistent that lower ones were considered indicative of the disease.

The daily course of the leucocyte counts of the 10 cats was plotted on chart A. It was found that the crisis occurred between the third and the tenth days with the mean

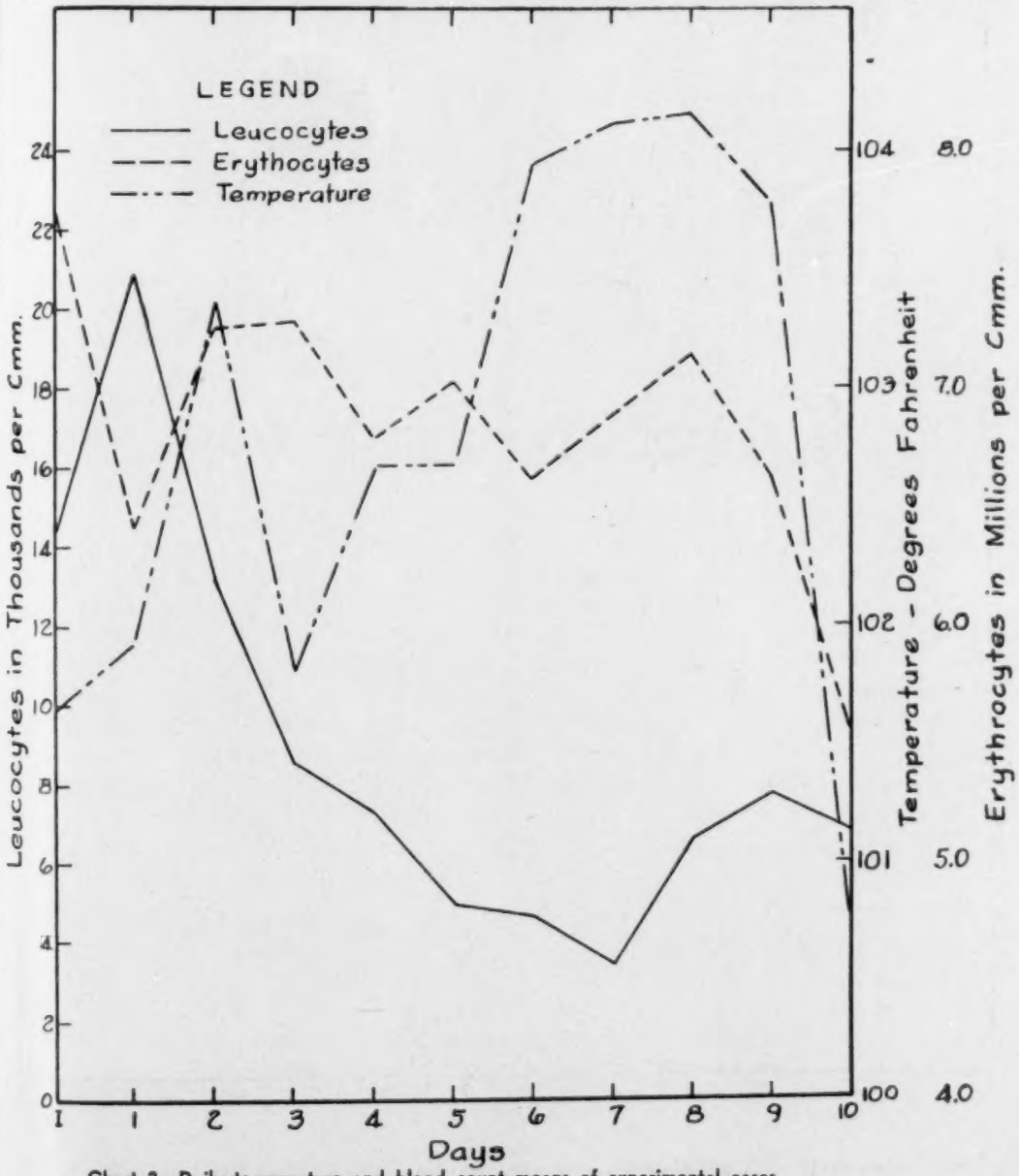

Chart 2—Daily temperature and blood count means of experimental cases.

TABLE 3—Means and Standard Deviation of the Fahrenheit Temperature of the Experimental Clinical Cases

Day	Cat 1	Cat 2	Cat 3	Cat 4	Cat 5	Cat 6	Cat 7	Cat 8	Cat 9	Cat 10	Standard Deviation	Means
1	101.0	102.5	101.8	100.5	101.8	102.5	100.8	102.0	101.8	101.8	.6774	101.65
1	102.0	102.5	103.0	102.0	101.0	100.6	102.9	101.8	101.4	101.6	.7775	101.88
2	105.5	104.4	105.0	106.0	102.5	101.5	102.4	102.2	102.5	101.8	1.6653	103.38
3	103.0	102.4	104.4	101.5	100.0	101.5	99.6D	102.0	102.0	101.5	1.3740	101.79
4	102.9	101.5	105.6	102.0	102.5	101.5		103.0	102.0	103.0	1.2510	102.67
5	105.3D	100.5	101.7D	102.9	101.8	99.8		103.0	106.0	103.0	2.0285	102.67
6		104.3		107.0D	101.8	103.9		102.0	106.0D	102.5	2.0062	103.93
7		105.0			101.4	105.8		106.3D		102.0	2.2494	104.10
8		104.5			102.0	105.6				104.5	1.5242	104.15
9		102.0			102.0R	103.8				107.0D	2.3580	103.70
10		100.0				101.2						
11		100.0D				101.0						
12						100.0						
13						101.0R						

I = Day of inoculation.
D = After last number in column means 'Death'.
R = After last number in column means 'Recovery'.

TABLE 4—Means and Standard Deviation of the Leucocyte Count of the Experimental Clinical Cases Using the Crisis Day as the Starting Point

Day	Cat 1	Cat 2	Cat 3	Cat 4	Cat 5	Cat 6	Cat 7	Cat 8	Cat 9	Cat 10	Standard Deviation	Means
10										15,100		
9										24,100		
8		11,500				14,950				16,600	2,602	14,350
7		17,400				10,100		13,050		12,100	3,081	13,162
6		10,200		16,200		10,000		28,800	10,500	15,300	7,212	15,167
5	15,400	6,000	17,000		10,800	8,200		16,000	18,000	11,700	4,379	12,888
4		4,200		21,200		4,600		10,200	15,800	10,800	6,555	11,133
3	11,300	6,800	10,000	20,800	15,000	6,200	17,500	5,300	10,050	6,250	5,291	10,920
2	5,200	2,400	1,350	18,800	8,900	6,450	27,000	6,750	3,600	8,900	8,009	8,935
1	6,900	350	800	5,400	6,500	2,400	6,750	3,450	4,200	10,200	3,060	4,695
C	700D	300	50D	600D	2,550	425	2,850D	600D	900D	400D	960	938
1		4,300			7,500	425					3,543	4,075
2		6,900			7,050	13,000					3,479	8,983
3		16,600D			16,500	26,700					5,860	19,933
4					15,700R	30,150					10,218	22,925
5						28,600R						

C = Crisis day.
D = After last number in column means 'Death'.
R = After last number in column means 'Recovery'.

of six and two tenths (6.2) days and a standard deviation of one and nine tenths (1.9) days. The chart also showed that the leucocytes increased from a preinoculation mean of 14,200 cells per cubic millimeter to 20,900 cells per cubic millimeter on the first postinoculation day. The leucocyte counts, from the first day to the crisis day, followed a rapidly downward course. The chart also shows that death usually took place near the crisis point and, if it did not occur then, a leucocyte increase was noted immediately.

The means and standard deviations of the daily variations of temperature, the leucocyte, and the erythrocyte counts for the 10 cases were calculated from tables 1, 2, and 3. They were plotted on chart B. This chart clearly shows the trend of the behavior of these three items during the course of the disease.

The temperatures rose sharply during the crisis period, while the leucocyte counts declined rapidly. The erythrocyte counts showed some variation early in the illness and, at the crisis, there was a marked progressive anemia. This picture, however, was misleading, because the leucocyte mean when calculated in this manner did not go below 3,330 cells per cubic millimeter on any given day. This was due to the individual variation of the time when the crisis occurred.

A more accurate picture of what occurred was demonstrated by using the crisis day of each case as the starting point, and figuring the variations in temperatures and blood counts in terms of the number of days before and after the crisis day. The results of such a comparison may be seen in tables 4, 5, and 6, and chart C.

Chart C, prepared from the means of tables 4, 5, and 6, brings out a definite correlation between the behavior of the leucocytes, the erythrocytes, and the temperatures. Chart B, prepared by using the inoculation day as the starting point, was subject to many irregularities caused by the marked individual variation of the cat's reaction to the material.

The lines on chart C were free from the irregularities shown on chart B. The leucocyte counts, for example, showed a marked regular daily decrease until the crisis was reached; then there was marked regular increase at about the same rate as seen in the previous decrease. The course of the erythrocyte count was one of marked decline and the trend was at almost the same rate as that of the leucocytes. There was, however, a slight increase on the crisis day (chart C). This may be explained on the basis of fluid loss, for all the animals were markedly dehydrated on their crisis day. They experienced varying degrees of pyrexia, vomition, diarrhea, and anorexia, and they refused to drink. The anemia, because of the marked body dehydration, was probably greater on all the days before the crisis than was actually recorded by the erythrocyte count. This is supported by the observation that the erythrocyte count did not reach its crisis point until three days after the crisis of the temperatures and leucocyte counts. The animals which survived drank immediately following the leucocyte crisis and temperature drop. Bone marrow studies by Lawrence *et al.*[1] also showed that all the blood elements were regenerated at their normal ratio. This suggests that new erythrocytes enter the peripheral blood at the same comparative rate as the leucocytes, but the hemoconcentration was so great at the crisis that, when this was relieved by fluid intake, it took three days before an erythrocyte mean was approximately the same as that of the leucocyte mean (chart C).

Chart C should be of great aid, not only in diagnosing panleucopenia, but also in determining the course that the disease is expected to follow. A differential count of the leucocytes will also be required to determine whether the infection is in the postcrisis or precrisis period. The precrisis differential counts showed no juvenile forms of neutrophils and only a few stab forms (2 to 5%). The percentage of neutrophils was low compared with the

other leucocytes (10 to 50%). In the post-crisis period, nearly 90 per cent of the cells were neutrophils, and a high percentage of the cells was juvenile in character.

SUMMARY

The disease has been shown to follow a rather consistent clinical course. Early in the onset, there is a slight elevation of temperature, retarded appetite, and rapid loss of body fluid and flesh. An animal may continue to play and respond normally to its surroundings until about the second to the sixth day after the temperature rises, when it becomes very depressed, dehydrated, and assumes a prone position (fig. 1). The temperature rises sharply and occasionally there is vomiting. It rises as the disease progresses, but immediately after

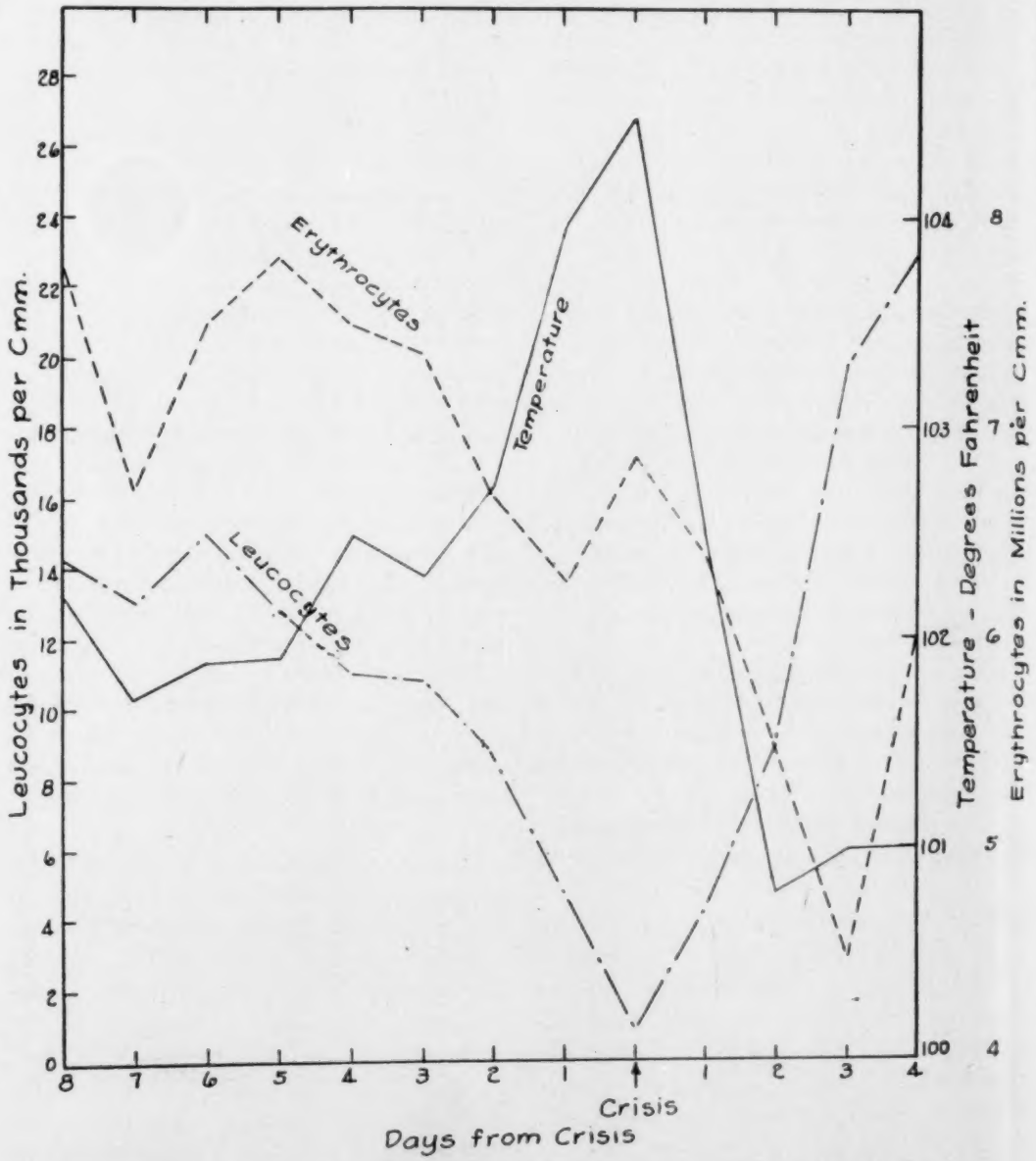

Chart 3—Daily temperature and blood count means of experimental cases by crisis days.

TABLE 5—Means and Standard Deviation of the Erythrocyte Count of the Experimental Clinical Cases Using the Crisis Day as the Starting Point

Day	Cat 1	Cat 2	Cat 3	Cat 4	Cat 5	Cat 6	Cat 7	Cat 8	Cat 9	Cat 10	Standard Deviation	Means
10										7.00		
9										6.72		
8		8.38				8.36				6.72	.9526	7.820
7		6.26				7.10		7.52		6.74	.1693	6.905
6		8.16		7.80		7.00		7.90	8.04	6.10	.7984	7.500
5	9.09	8.34	9.22		6.72	7.08		8.74	6.22	7.20	1.1583	7.826
4		8.17		6.99		6.90		8.34	7.06	7.60	.6289	7.510
3	7.08	8.64	7.38	6.84	7.96	7.12		7.96	6.80	6.76	.6547	7.393
2	7.20	8.27	7.04	6.28	7.00	7.32	5.10	6.90	6.65	7.08	.6621	6.684
1	6.49	7.44	5.56	5.50	6.72	7.92	4.40	6.22	6.20	6.50	.9994	6.295
C	9.00D	8.56	6.68D	5.80D	6.02	7.36	5.50D	6.34D	7.06D	6.08D	1.1712	6.840
1		6.66			5.66	6.94					.6729	6.420
2		4.41			5.90	6.10					.9234	5.470
3		4.27D			5.60	3.34					1.1359	4.403
4					6.26R	5.76					.3536	6.010
5						7.07R						

C = Crisis day.
D = After last number in column means 'Death'.
R = After last number in column means 'Recovery'.

TABLE 6—Means and Standard Deviation of the Fahrenheit Temperature of the Experimental Clinical Cases Using the Crisis Day as the Starting Point

Day	Cat 1	Cat 2	Cat 3	Cat 4	Cat 5	Cat 6	Cat 7	Cat 8	Cat 9	Cat 10	Standard Deviation	Means
9										101.8		
8		102.5				102.5				101.6	.5196	102.20
7		102.5				100.6		102.0		101.8	.8057	101.72
6		104.4		100.5		101.5		101.8	101.8	101.5	1.3076	101.92
5	101.0	102.4	101.8	102.0	101.8	101.5		102.2	101.4	103.0	.5916	101.90
4	102.0	101.5	103.0	106.0	101.0	101.5		102.0	102.5	103.0	1.4790	102.50
3	105.5	100.5	105.0	101.5	102.5	99.8	100.8	103.0	102.0	102.5	1.8430	102.31
2	103.0	104.3	104.4	102.0	100.0	103.9	102.9	103.0	102.0	102.0	1.3269	102.75
1	102.9	105.0	105.6	102.9	102.5	105.8	102.4	102.0	106.0	104.5	1.5728	103.96
C	105.3D	104.5	101.7D	107.0D	101.8	105.6	99.6D	106.3D	106.0D	107.0D	2.5599	104.48
1		102.0			101.8	103.8					1.1015	102.53
2		100.0			101.4	101.2					.7572	100.87
3		100.0D			102.0	101.0					1.00	101.00
4					102.0R	100.0R					1.00	101.00

C = Crisis day.
D = After last number in column means 'Death'.
R = After last number in column means 'Recovery'.

the crisis, there is a rapid drop toward the normal.

The disease can be accurately and easily diagnosed by the aid of blood counts, for, early in this disease, the bone marrow becomes aplastic and all the cellular elements of the peripheral blood diminish. The decrease in blood cells, however, is the most noticeable in the leucocytes, because in many cases they almost or entirely disappear at the crisis. Ailing, adult cats with leucocyte counts below 10,000 cells per cubic millimeter and kittens with counts below 8,000 cells per cubic millimeter should be considered as possible early cases.

The course may be quite accurately determined if daily temperature, leucocyte, and erythrocyte determinations are plotted and compared with chart C.

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[2]Hammon, W. D., and Enders, J. F.: A Virus Disease of Cats, Principally Characterized by Aleucocytosis, Enteric Lesions and the Presence of Intranuclear Inclusion Bodies. J. Expti. Med., *69*, (1939): 327-352.

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Chicken Embryo Vaccine Against Newcastle Disease [Pneumoencephalitis]

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NEWCASTLE DISEASE [pneumoencephalitis] otherwise known as avian pest, Doyle's disease, Ranikhet, or pseudofowl pest was first reported by Doyle[1] to have occurred in Newcastle-on-Tyne in 1926. According to Farinas[2], it was first observed in this country in September, 1927. The disease has spread rapidly among the chicken population of the country, killing hundreds of thousands of them and, after about thirteen years of existence, it has invaded practically all regions of the archipelago. To this virus disease is attributed the failure of many poultry projects in the country. It has become the greatest hindrance to the campaign of the Bureau of Animal Industry for the self-sufficiency in poultry and egg supply. Hence, the control of this disease has been the subject of study continually for the last thirteen years.

The development of a biological product that can be effectively used for the control of the disease has been the subject of studies since the discovery of its presence in this country. Farinas[2] prepared and tried 17 types of vaccines from various organs or combinations of organs of infected chickens treated with phenol, tricresol, and chloroform, with variable results. Topacio and Coronel[3] prepared and tried 23 different kinds of vaccine from almost every organ excepting the muscles and skin of the infected chickens and used as attenuating substances 13 different chemicals and dyes which possessed antiseptic properties. As their results were unsatisfactory, they concluded that there seems to be no way in sight of converting the crop virus or organs of an infected chicken into a product with immunizing properties, and suggested the adaptation of Newcastle virus in mammals such as rabbits, guinea pigs, rats, and mice, so as to be able to produce a vaccine like the one used against rabies.

Presented before the twenty-fourth annual congress of the Philippine Veterinary Medical Association, Feb. 21, 1941.

From the Pathology and Bacteriology Division, Bureau of Animal Industry, Manila, Philippine Islands.

We express our gratitude to Dr. Gregorio San Agustin, director of the Bureau of Animal Industry, for, without his unfaltering faith and interest in this work, it could not have been accomplished. We are equally indebted to Dr. Juan D. Generoso, chief of the Pathology and Bacteriology Division, for his encouragement and valuable suggestions. We thank Dr. Vincente Ferriols, chief of the Animal Disease Control Division, for his sincere interest and full support made possible the field trials of the vaccine. Last, but not least, we are indebted to Dr. Torcuato F. Rivadelo, supervising veterinarian, Animal Disease Control Division, for, without his openmindedness and untiring efforts, the field trials of the vaccine could not have been successfully undertaken.

REVIEW OF THE LITERATURE

The writers lament the scarcity of literature in our libraries dealing with the progress of the investigations in other countries on the development of vaccines against this disease. According to available literature, it appears that Doyle[1], Cooper[4], and Picard[5] have attempted to prepare vaccines. Doyle's heat-killed and formolized vaccine was only able to produce a slight increase in resistance among the inoculated birds, and Cooper's vaccine, consisting of tissue of infected chickens treated with chloroform, proved valueless. Picard, using a formolized, exploded tissue vaccine, failed to produce any protection against the disease.

The significant findings of Higbie and Howitt[6] that the virus of equine encephalomyelitis grows well in the developing chicken embryo, coupled with the equally important observations of Beard, Finkelstein, Sealy, and Wyckoff[7] that the two types of encephalomyelitis viruses not only grow well in the developing chicken embryo but also multiply in vast proportion (100,000 to 100,000,000 times the amount found in horse brain), led Dr. Juan D. Generoso, then assistant chief

of the erstwhile veterinary research division of the local Bureau of Animal Industry, to believe that the same would apply to Newcastle disease since it is primarily a disease of chickens.

Immediately, work along this line was started. Generoso, Acevedo, and Mendoza[8] found that the virus of Newcastle disease multiplied in 9-day-old chicken embryos as well as in 11-day-old duckling or turkey embryos from 20,000 to 40,000 times the amount of virus found in the crop, of which 1 cc. contained sufficient virus to infect or kill 1,000,000 chickens as previously determined by Topacio[9]. Another important finding noted is the existence of the virus, in practically

TABLE I—Summary of Material Used, Method of Attenuation, Safety and Potency Test of the Different Experimental Vaccines Studied

Vaccine no.	Material used	Method of attenuation	Birds used for safety and potency test	Amt. vaccine injected	Results	Remarks
1	10% turkey embryo in glycerinated buffer solution*	0.05% formalin 48 hr. at 25 C.	1 Rhode Island Red cockerel 4-5 mo. old	2cc.	I.	D.
2		0.1% formalin 3 da. in elect. refrigerator	1 Rhode Island Red cockerel 4-5 mo. old	2cc.	I.	D.
3		0.2% formalin 3 da. in elect. refrigerator	1 Cantonese cockerel 4-5 mo. old	2cc.	I.	D.
4		0.3% formalin 3 da. in elect. refrigerator	1 Rhode Island Red cockerel 4-5 mo. old	2cc.	I.	D.
5		0.4% formalin 3 da. in elect. refrigerator	1 Rhode Island Red cockerel 4-5 mo. old	2cc.	I.	D.
6		1:40,000 Merthiolate 48 hr. at 25 C.	1 Cantonese cockerel 4-5 mo. old	2cc.	I.	D.
7		1:50,000 Merthiolate 48 hr. at 25 C.	1 Rhode Island Red cockerel 4-5 mo. old	2cc.	I.	D.
8		1:60,000 Merthiolate 48 hr. at 25 C.	1 Rhode Island Red cockerel 4-5 mo. old	2cc.	I.	D.
9		1:70,000 Merthiolate 48 hr. at 25 C.	1 Rhode Island Red cockerel 4-5 mo. old	2cc.	I.	D.
10		1:80,000 Merthiolate 48 hr. at 25 C.	1 Rhode Island Red cockerel 4-5 mo. old	2cc.	I.	D.
11		0.5% formalin 5 da. at 25 C.	1 White Leghorn cockerel 4-5 mo. old	2cc.	I.	D.
12		0.4% formalin 10 da. in elect. refrigerator	1 White Leghorn cockerel 4-5 mo. old	2cc.	I.	D.
13		1:40,000 Merthiolate 48 hr. at 25 C. 48 hr. at room temp.	1 White Leghorn cockerel 4-5 mo. old	2cc.	I.	D.
14		1:40,000 Merthiolate 2 da. at room temp.	1 White Leghorn cockerel 4-5 mo. old	2cc.	I.	D.
15		0.15% tricresol 24 hr. at 37 C.	1 White Leghorn cockerel 4-5 mo. old	2cc.	I.	D.
16		0.3% tricresol 24 hr. at 37 C.	1 White Leghorn cockerel 4-5 mo. old	2cc.	I.	D.
17		0.2% phenol 24 hr. at 37 C.	1 White Leghorn cockerel 4-5 mo. old	2cc.	I.	D.
18		0.5% phenol 24 hr. at 37 C.	1 White Leghorn cockerel 4-5 mo. old	2cc.	I.	D.
19		1:40,000 Merthiolate 2 da. at 25 C. 2 days at room temp. 1 da. at 37 C.	1 White Leghorn cockerel 4-5 mo. old	2cc.	I.	D.

*Composition of glycerinated buffer solution:
M/15 dibasic sodium phosphate solution..... 80.8 cc.
M/15 monobasic potassium phosphate solution..... 19.2 cc.
Glycerin (pH 7.2 after sterilization)..... 100.0 cc.

I = infective. D = discarded.
A = attenuated. Protective against 4 m.l.d.'s of amnionic fluid virus.

the same concentration, in the embryo proper, the chorioallantoic membrane, and the amnionic fluid.

CULTIVATION OF NEWCASTLE DISEASE VIRUS

The method used for the cultivation of the virus is essentially that of Burnet[19] with slight modifications which we have adapted in connection with this work. Chicken eggs are incubated nine days or, when duck or turkey eggs are used, they are incubated eleven to twelve days and examined by transillumination to learn if the embryos are alive. The fertile eggs are then washed with warm 3 per cent chlorazene solution and dried with sterile gauze. A small triangular area is drilled on a side of the shell of each egg and the

TABLE I (Continued)—Summary of Material Used, Method of Attenuation, Safety and Potency Test of the Different Experimental Vaccines Studied

Vaccine no.	Material used	Method of attenuation	Birds used for safety and potency test	Amt. vaccine injected	Results	Remarks
20	10% turkey embryo in glycerinated buffer solution	1:60,000 Merthiolate 24 hr. at 37 C	1 White Leghorn cockerel 4-5 mo. old	2cc.	I.	D.
21		0.05% formalin 48 hr. at room temp	1 White Leghorn cockerel 4-5 mo. old	2cc.	A.	T.‡
22		1:80,000 Merthiolate 2 da. at 25 C. 2 da. at room temp. 1 da. at 37 C.	1 White Leghorn cockerel 4-5 mo. old	2cc.	I.	D.
23		0.05% formalin 7 da. at 25 C.	1 White Leghorn cockerel 4-5 mo. old	2cc.	A†	T.‡
24		1:40,000 Merthiolate 48 hr. at room temp	Barred Plymouth rooster Barred Plymouth hen	2cc.	I.	D.
25	10% duck embryo in glycerinated buffer solution	0.05% formalin 3 da. at room temp.	1 White Leghorn cockerel 4-5 mo. old	2cc.	A‡	D.
26		1% sodium ricinoleate 48 hr. at room temp.	1 White Leghorn cockerel 4-5 mo. old	2cc.	A‡	D.
27		2% sodium ricinoleate 48 hr. at room temp.	1 White Leghorn cockerel 4-5 mo. old	2cc.	A‡	D.
28	10% turkey chorioallantoic membrane in glycerinated buffer solution	0.05% formalin 3 days at room temp.	1 White Leghorn cockerel 4-5 mo. old	2cc.	A‡	D.
29	10% duck embryo in glycerinated buffer solution	0.05% phenol 48 hr. at 37 C.	1 White Leghorn cockerel 4-5 mo. old	2cc.	A‡	D.
30		0.3% tricresol 48 hr. at 37 C.	1 White Leghorn cockerel 4-5 mo. old	2cc.	A‡	D.
31		1:20,000 Merthiolate 4 da. at room temp.	1 White Leghorn cockerel 4-5 mo. old	2cc.	I.	D.
32	10% turkey embryo in glycerinated buffer solution	0.25% sod. ricinoleate 48 hr. at room temp.	1 White Leghorn cockerel 4-5 mo. old	2cc.	I.	D.
33		0.05% sod. ricinoleate 48 hr. at room temp.	1 White Leghorn cockerel 4-5 mo. old	2cc.	A‡	D.
34	10% chicken embryo in glycerinated buffer solution	0.05% formalin 3 da. at room temp.	2 Rhode Island Red cockerels 1 White Leghorn cockerel	2cc.	A‡	D.

A†=attenuated. Failed to protect against 1,000 m.i.d.'s of crop virus.
A‡= attenuated. Failed to protect against 20 m.i.d.'s of amnionic fluid virus.
T‡= Test dose of virus too small. T‖= Test dose of virus too heavy.
The controls used in these trials of experimental vaccines which were given 1 m.i.d. of virus invariably developed the disease and died or recovered.

TABLE 2—Summary of Vaccines Prepared With Higher Concentrations of Tis sue and Incorporating the Chorioallantoic Membranes and Amnionic Fluids

Vaccine no.	Material used	Attenuation method	Birds used Safety and potency test	Am. vaccine injected	Results	Remarks
35	6.5% chicken embryo, 6.5% membrane, 12.5% amnionic fluid in glycerinated buffer solution*	20 min. at 60 C.	1 W. L. C.	2 cc.	Attenuated. Protected against 20 m.i.d.'s amnionic fluid virus	H.C.†
36		30 min. at 60 C.	1 W. L. C.	2 cc.	Attenuated. Protected against 20 m.i.d.'s amnionic fluid virus	H.C.†
37		40 min. at 60 C.	4 W. L. C. injected 11 da. after vaccine prepared	2 cc.	Attenuated. 3 withstood 20 m.i.d.'s amnionic fluid virus	75% P.
38		30 min. at 60 C. 1:50,000 Merthiolate as preservative	2 W. L. C.	2 cc.	Attenuated. All survived 20 m.i.d.'s amnionic fluid virus	100% P.
39		30 min. at 60 C. 1:60,000 Merthiolate as preservative	2 W. L. C.	2 cc.	Attenuated. All survived 20 m.i.d.'s amnionic fluid virus	100% P.
40		0.05% formalin 4 da. at room temp.	2 W. L. C.	2 cc.	Attenuated. All survived 20 m.i.d.'s amnionic fluid virus	100% P.
41		0.7% sodium ricinoleate 4 da. at room temp.	2 W. L. C.	2 cc.	Attenuated. All survived 20 m.i.d.'s amnionic fluid virus	100% P.
42		0.7% sodium borate 4 da. at room temp.	2 W. L. C.	2 cc.	Attenuated. All survived 20 m.i.d.'s amnionic fluid virus	100% P.
43		1:2,000 crystal violet 9 da. in ordinary ice box	2 W. L. C.	2 cc.	Attenuated. All survived 20 m.i.d.'s amnionic fluid virus	100% P.
44		1:2,000 crystal violet 2 da. at 37 C.	2 W. L. C.	2 cc.	Attenuated. All survived 20 m.i.d.'s amnionic fluid virus	100% P.
45		1:2,000 crystal violet 0.7% sodium borate 9 da. in ice box	2 W. L. C.	2 cc.	Attenuated. All survived 20 m.i.d.'s amnionic fluid virus	100% P.
46		1:2,000 crystal violet 0.7% sodium borate 2 da. at 37 C.	2 W. L. C.	2 cc.	Attenuated. All survived 20 m.i.d.'s amnionic fluid virus	100% P.
47	1 part chicken embryo, membrane, and amnionic fluid mixture and 1 part glycerinated buffer solution	1:2,000 crystal violet 0.7% sodium borate 10 da. in ice box	2 W. L. C.	1 cc.	Attenuated. All withstood 20 m.i.d.'s amnionic fluid virus	100% P.
48		0.05% formalin 10 da. in ice box	4 W. L. C.	1 cc.	Attenuated. All withstood 100 m.i.d.'s amnionic fluid virus	100% P.
49		0.06% formalin 10 da. in ice box	1 W. L. C.	1 cc.	Attenuated. Withstood 100 m.i.d.'s of virus	100% P.
50		0.7% formalin 10 da. in ice box	1 W. L. C.	1 cc.	Attenuated. Withstood 100 m.i.d.'s of virus	100% P.
51		0.09% formalin 10 da. in ice box	1 W. L. C.	1 cc.	Attenuated. Withstood 100 m.i.d.'s of virus	100% P.
52		0.1% formalin 10 da. in ice box	4 W. L. C.	1 cc.	Attenuated. All cockerels survived 50-100 m.i.d.'s of virus	100% P.
			10 chicks 2 mo. old	0.5 cc.	6 chicks were protected against 50 m.i.d.'s of virus. 4 chicks not protected.	60% P.
53	1 part chicken embryo, membrane, and amnionic fluid mixture and 1 part glycerinated buffer solution	0.2% formalin 10 da. in ice box	2 W. L. C.	1 cc.	Attenuated. Survived 10-50 m.i.d.'s of virus	100% P.

53	1 part chicken embryo, membrane, and aminonic fluid mixture and 1 part glycerinated buffer solution	0.2% formalin 10 da. in ice box	2 W. L. C.	1 cc.	Attenuated. Survived 10-50 m.l.d.'s of virus	100% P.
54		1:2,000 crystal violet and 0.05% formalin 10 da. in ice box	4 W. L. C.	1 cc.	Attenuated. All survived 50 m.l.d.'s of virus	100% P.
55		0.7% sodium borate 0.05% formalin 10 da. in ice box	2 W. L. C.	1 cc.	Infective	D.
57	1 part duck embryo, membranes, and amnionic fluid mixture with glycerinated buffer solution	0.1% formalin 10 da. in ice box	2 W. L. C.	1 cc.	Attenuated. Cockerels were protected against 100 m.l.d.'s of virus.	100% P.
			10 chicks 2 mo. old	0.5 cc.	5 chicks protected against 50 m.l.d.'s of virus. 5 chicks not protected.	50% P.

The controls used in these trials of experimental vaccines which were given 1 m.l.d. of virus invariably developed the disease and died or recovered.

*Composition of glycerinated buffer solution:

M/15 dibasic sodium phosphate solution ..... 80.8 cc.
M/15 monobasic potassium phosphate solution ..... 19.2 cc.
Glycerin (pH 7.2 after sterilization)..... 100.0 cc.

†H.C. = Higher concentration of vaccine material seemed to give protection. W.L.C. = White Leghorn cockerel. P=Protection. D.=Discarded.

dust removed by pumping air on it from an air compressor. We have found it no longer necessary to drill a hole through the air sac in order to create an air space beneath the drilled triangular area. At the time of inoculation, the triangular piece is lifted off with the aid of a fine-bladed scalpel, and 0.05 to 0.1 cc. of amnionic fluid virus is injected through the external membrane and into the chorioallantoic membrane by means of a tuberculin syringe with a 27-gauge needle. The triangular openings are then sealed with sealing wax, and the eggs are returned to the incubator.

After twenty-four to thirty-six hours of incubation, the eggs are opened and the infected chicks, their chorioallantoic membranes, and amnionic fluids are removed from the shell. The amnionic fluid is aspirated aseptically with a syringe and collected into sterile bottles. The embryos and membranes are collected in graduated cylinders so that their volumes can be determined readily. These materials are stored in the refrigerator until they are used for vaccine preparation.

TITRATIONS OF THE VIRUS CONTENTS

Using 1 cc. as the dose and injecting subcutaneously into 4- to 5-month-old White Leghorns (having found this breed to be most susceptible), we have determined that the amount of virus contained in these materials was infective, if not lethal, in the following dilutions:

Chicken embryo	1 : 20,000,000,000
Chicken embryo chorioallantoic membrane	1 : 10,000,000,000
Chicken embryo amnionic fluid	1 : 40,000,000,000
Duck embryo	1 : 20,000,000,000
Duck embryo amnionic fluid ..	1 : 20,000,000,000
Turkey embryo	1 : 40,000,000,000
Turkey embryo chorioallantoic membrane	1 : 10,000,000,000
Turkey embryo amnionic fluid	1 : 40,000,000,000

It has been determined by these titrations that the infected chicken, duck, or turkey embryos, their membranes, and amnionic fluids contained from 20,000 to

40,000 times more virus than the amount found in the crop contents of infected chickens, of which 1 cc. contains sufficient virus to infect 1,000,000 chickens. These titrations were described in a paper by Generoso, Acevedo, and Mendoza[8]. Frequent determinations of the concentration of the virus in these materials, which to date has already mounted to 50 passages, have shown that they have not changed.

EXPERIMENTAL VACCINES

Following closely the method of Mitchell, Walker and Plummer[11] in the preparation of the equine encephalomyelitis vaccine, 10 per cent emulsions of infected embryos alone and of purely allantoic membrane were made in a vehicle composed of M/15 dibasic sodium phosphate and M/15 monobasic potassium phosphate solution and glycerin (50%) with a pH of about 7.2 after sterilization. Small batches of the tissue emulsions, treated with different chemicals and exposed to different physical conditions as shown in table 1, were tested for immunity producing properties.

Having failed to produce a protective vaccine with the 10 per cent emulsion of embryo, we deviated from the method advocated by Mitchell, Walker, and Plummer[11] by using heavier concentration of tissue, and included the allantoic membranes and amnionic fluids in the preparation of the succeeding vaccines studied. These vaccines are described in table 2.

Besides the vaccines which were described in tables 1 and 2, we have tried drying the ground, infected embryo, together with the membranes and amnionic fluids, over sulfuric acid at room temperature and over calcium chloride in an ice chamber. The material dried over sulfuric acid was found noninfective only after a hundred days' exposure at room temperature. However, 0.2 to 0.5 Gm. of this dried, noninfective tissue did not protect chickens from 20 m.i.d.'s of virus. The tissue dried over calcium chloride and stored in the ice chamber was found infective even after nine months, so that this material also can not be utilized for vaccine purposes until a method of altering its virulence is worked out.

Among the vaccines which gave promising results, vaccine 47, treated with crystal violet and sodium borate, was picked to be tested further because it did not contain formalin. We feared that the formolized vaccines 48 to 52, which proved good, might not keep as long as is true of other formolized biological products. So,

TABLE 3—Determination of Protective Properties of Crystal Violet and Sodium Borate-Treated Vaccine 47 against Inoculation of Virus

White Leghorn cockerel no.	Vaccination date	Amt. vaccine given	Challenge inoculation	Date injection of challenge inoculation	Results
479	7-7-40	1 cc.	100 m. i. d.'s amnionic fluid virus	7-16-40	Survived
480	7-7-40	1 cc.	100 m. i. d.'s amnionic fluid virus	7-16-40	Survived
481	7-7-40	1 cc.	100 m. i. d.'s amnionic fluid virus	7-16-40	Survived
666	7-7-40	1 cc.	100 m. i. d.'s amnionic fluid virus	7-16-40	Survived
676	7-7-40	1 cc.	100 m. i. d.'s amnionic fluid virus	7-16-40	Survived
Control	....	....	1 m. i. d.	7-16-40	Died of Newcastle disease 10-22-40

12 White Leghorn cockerels were injected with vaccine 47. Two of the vaccinated birds died of roup. Fifteen days after the vaccination, the remaining chickens were divided into two groups. One group was given 100 m.i.d.'s of virus and the other group was exposed in a cage together with 2 infected chickens and 2 nonvaccinated controls. None of the 10 vaccinated birds contracted the disease, as shown in tables 3 and 4.

TABLE 4—Determination of Protective Properites of Crystal Violet Vaccine 47 Against Natural Exposure.

White Leghorn cockerel no.	Date Vaccination	Amt. vaccine used	Date of exposure	Results
482	7-1-40	1 cc.	7-18-40	Survived
483	7-1-40	1 cc.	7-18-40	Survived
484	7-1-40	1 cc.	7-18-40	Survived
1717	7-1-40	1 cc.	7-18-40	Survived
1789	7-1-40	1 cc.	7-18-40	Survived
1954	7-1-40	1 cc.	7-18-40	Survived
Control 1	....	....	7-18-40	Died of Newcastle disease 7-25-40
Control 2	.....	....	7-18-40	Died of Newcastle disease 7-29-40
Infected cockerel 1	.....	....	.....	Died 7-20-40
Infected cockerel 2	.....	....	.....	Died 7-21-40

After three months' storage in the refrigerator, the crystal violet-sodium borate, chicken embryo vaccine 47 was tested again to determine its keeping qualities. Four White Leghorn cockerels were used, and the results will be found in table 5.

FIELD APPLICATIONS OF THE VACCINE

Due to the urgent necessity of having something which could be tried against the outbreaks of Newcastle disease on the poultry farms of Manila and the nearby provinces, which assumed serious proportion starting from December, 1940, a vaccine was prepared in the following manner:

Serial 1.—Equal parts of 9-day-old, infected chicken embryos, their allantoic membranes and amnionic fluids, and sterile, glycerinated buffer solution (M/15 dibasic phosphate, 80.8 cc.; M/15 monobasic potassium phosphate, 19.2 cc.; glycerin, 100 cc.) standardized to crystal violet 1 : 2,000 (0.05%) and sodium borate, 0.7 per cent.

These materials were ground in a colloidal mill for forty minutes. After grinding, the preparation was filtered through four thicknesses of sterile gauze into a 5-liter sterile bottle and placed in an ordinary ice box maintaining a temperature of 18 to 22 C. It was kept in the ice box for ten days and shaken for four minutes every day.

Serial 2.—Prepared in the same manner as serial 1, using 11-day-old duck embryos instead of chicken embryos. The same strengths of crystal violet and sodium borate were used.

For causes which we could not determine, the two preparations were found still infective after ten days' storage in an ice box. This was contrary to our experience with our experimental, crystal violet-sodium borate vaccine 47. However, our results with experimental vaccines 48 to 53 which were treated with formalin, ranging from 0.05 to 0.2 per cent, showed beyond question that this substance at these concentrations did not affect the protective properties of the vaccine. We, therefore, added 0.1 per cent formalin to serial 1 and 0.05 per cent to serial 2. After the addition of these amounts of formalin, the products were found safe to use.

Due to the scarcity of susceptible chickens only 4 were used for safety and potency tests of these two serials of vaccine, 2 White Leghorns and 2 Rhode Island Reds, 1 of each breed for each serial. The two serials of vaccine proved to be safe and sufficiently potent to protect fully all the test birds against 50 to 100 m.i.d.'s of amnionic fluid virus.

The dose of the vaccine used in the field was 1 cc. for adults and 0.5 cc. for chicks over 2 months old. The vaccine was injected intramuscularly on the lateral side of the leg. We did not attempt to vaccinate chicks less than 2 months old in view of the rather poor response to vaccination of 2-month-old chicks with vaccines 52 and 56. The poor response by 2-month-old chicks to the vaccination may be attributed to the fact that in young animals the reticulo-endothelial system, which is the body tissue responsible for antibody production, was not yet well developed.

The vaccine was first tried in Malolos, Bulacan, on Dec. 28, 1940, in an outbreak of the disease among native stock. A total of 252 chickens were vaccinated, consisting of 183 White Leghorns, 25 Texas Game cocks and hens, and 48 native chickens. The vaccination checked the infection among native stocks and protected two flocks of White Leghorns. Of the 252 chickens vaccinated, only 2 died of Newcastle disease and 1 developed the chronic form of the disease; about 98 per cent were protected.

Encouraged by the good results obtained in Malolos, Bulacan, the vaccine was then tried in the poultry owned by the Central Luzon Milling Corporation of Bamban, Tarlac, where 80 White Leghorns had died of the disease prior to vaccination. The following flocks were vaccinated:

Black Minorcas	13	(apparently free)
Nagoyas	52	(some showing symptoms of the disease)
Cantonese	31	(apparently free)
Rhode Island Reds	43	(apparently free)
White Leghorns	21	(very badly infected)
White Leghorn chicks (2-3 months old)	54	(apparently free)

The following were not vaccinated:

White Leghorns	20	(very badly infected)
White Leghorn chicks (1-2 months old)	100	(apparently free)

Of the 87 chickens which were apparently free from infection, only 3 came down with the disease, or about 96 per cent were protected. Thirty-nine of the 54 vaccinated birds died of roup as did the 100 unvaccinated ones. Of the 73 birds belonging to the infected flock, 25 survived. Of these, 22 were Nagoyas and 3 were White Leghorns, or about 40 per cent of the Nagoyas and about 13 per cent of the White Leghorns were saved. Of 20 infected White Leghorns which were not vaccinated, 2 survived, or 90 per cent succumbed to the infection.

An outbreak of Newcastle disease occurred in the poultry of Trinidad Stock Farm in Baguio, where 500 birds died of the disease. The vaccine was also tried there. The following flocks were vaccinated:

White Leghorns	171	(apparently free)
White Leghorn chicks	34	(apparently free)
Rhode Island Reds	85	(apparently free)
Total	290	
White Leghorns	66	(very badly infected)
White Leghorn chicks (2-3 months old)	113	(affected with roup and Newcastle disease)
Total	179	

Among the 290 supposedly unaffected birds, 6 showed signs of the disease, but 5 of them got well after two or three days of illness; 1 died, or about 98 per cent were protected. Only 6 adult White Leghorns survived of the 179 which were actually infected when the vaccine was given. It is clearly shown in this observation that the vaccine does not have any curative value.

Newcastle disease broke out in the flock of Rudolfo Santos of Santa Maria, Bulacan. He has only one poultry house which is divided into three compartments, separated by poultry-wire netting. In the compartment at the left, 40 young pullets were housed; in the middle compartment were the laying flock of 40 hens and a few

roosters; and in the compartment at the right were 34 pullets beginning to lay. When disease which broke out in the middle compartment was brought to our attention, two hens had already died. Before vaccination, we removed to an isolation pen 6 hens showing symptoms of the disease and 3 more soon after vaccination. The following vaccinations were made:

Young pullets	40	(apparently free)
Laying pullets	34	(apparently free)
Laying hens and roosters	32	(apparently free)
Total	106	

Of the young pullets which were apparently free from infection, none became infected, or 100 per cent were protected. Of the 32 birds in the infected unit, only 1 died, or 97 per cent were saved by the vaccine. All the chickens of the flocks within the radius of from 200 yards to about 1 kilometer from the infected poultry were vaccinated. More than 2,000 chickens were vaccinated, mostly White Leghorns, without experiencing any break or serious untoward effects except a few cases of lameness because of the practice of injecting the vaccine in the thigh instead of in the breast muscle, and a few cases of ruptured yolk due to rough handling in catching the hens. The vaccination was done in January and, one month later, no cases of the disease had been reported among the vaccinated chickens in that town.

Another case in which the vaccine showed its high efficiency in checking an early infection of Newcastle disease was in a flock of 23 White Leghorns, belonging to Bonifacio Perez of Barrio Santa Clara, Santa Maria, Bulacan. One of his pullets developed symptoms of the disease. It was shown to the senior author on one of his trips to that town. The sick bird was brought to Manila for observation, but it died on the way. The autopsy revealed the lesions of Newcastle disease. The remaining 22 birds were vaccinated while the infection was in its early stage. All of them were saved. Up to one month after vaccination, no more cases of Newcastle disease were reported from this flock.

Another instance in which the vaccine was applied with encouraging results was in Taytay, Rizal, in Muson poultry owned by a Japanese. In this place, the vaccine together with individual segregation worked very satisfactorily. Of the 597 birds vaccinated, 422 were apparently free and 150 were infected and were confined in an elevated quarantine pen that was provided with compartments to accommodate 2 to 4 chickens. The droppings from this pen were protected with wire netting. Among the 442 supposedly unaffected birds, not 1 developed the disease, or 100 per cent were protected. Of the 150 birds in the

TABLE 5—Determination of the Keeping Qualities of Crystal Violet-Sodium Borate Vaccine 47

White Leghorn cockerel no.	Vaccination date	Amt. vaccine given	Challenge inoculation	Date injection of challenge inoculation	Results
1	9-18-40	1 cc.	100 m.i.d.'s amnionic fluid virus	10-30-40	Survived
2	9-18-40	1 cc.	100 m.i.d.'s amnionic fluid virus	10-30-40	Survived
3	9-18-40	1 cc.	100 m.i.d.'s amnionic fluid virus	10-30-40	
4	9-18-40	1 cc.	100 m.i.d.'s amnionic fluid virus	10-30-40	
5 (control)	—	—	1 m.i.d.	10-30-40	Died of Nc disease 10-10-40

infected pens, 12 died of Newcastle disease, or 92 per cent were saved.

It would be interesting to mention here that, fourteen days after the vaccination, the birds which survived the infections were released from the quarantine pens by the owner and mixed with the clean, but vaccinated, birds. This was a dangerous step to take if his chickens were not immunized, because the released birds having just recovered from the disease might still have been eliminating some virus in their secretions. No case of the disease was again reported from his farm.

The results of these vaccinations are summarized in table 6.

There is one objectionable feature of the vaccine which we observed in its application in the field. The crystal violet stains the hands and clothing of the vaccinator.

Experiments are being conducted to eliminate the crystal violet. Previous trials have shown that sodium borate and formalin do not affect the antigenic pro-

TABLE 6—Summary of Results of Field Use of Vaccine

Name of place and owners of poultry	Flock no. and breed†	Status of flock before vaccination	No. deaths due to Nc* before vaccination	No. vaccinated	Results		
					Died of Nc	Unaffected	Protection (%)
Malolos, Bulacan							
Calalang	1. N.	Seriously infected	20	18	1	17	94
Joson	2. N.	Apparently free	0	14	2	12	85
Guzman	3. N.	Apparently free	0	8	0	8	100
R. Agustin	4. N.	Apparently free	0	4	1	3	75
Gonzales	5. W. L.	Free but in focus of infection	0	11	0	11	100
Dr. R. Reyes	6. T.		0	21	0	21	100
Dr. R. Reyes	7. W.		0	172	0	172	100
Bamban, Tarlac							
Central Luzon Milling Corp.	1. M.	Apparently free	0	13	3	10	84
	2. Na.	Infected	0	52	30	22	40
	3. C.	Apparently free	0	31	0	31	100
	4. R. I. R.	Apparently free	0	43	0	43	100
	5. W. L.	Seriously infected	80	21	18	3	13
Baguio, Mt. Province							
Trinidad Stock Farm	1. W. L.	Apparently free	0	171	0	171	100
Trinidad Stock Farm	2. W. L.	Apparently free	0	34 chicks	0	34	100
Trinidad Stock Farm	3. R. I. R.	Apparently free	0	85	6	79	93
Trinidad Stock Farm	4. W. L.	Seriously infected	500	66	60	6	10
Sta. Maria, Bulacan							
Rodolfo Santos	1. W. L.	Apparently free	0	40	0	40	100
Rodolfo Santos	2. W. L.	Apparently free	0	34	0	34	100
Rodolfo Santos	3. W. L.	Infected	2	32	1	31	97
Various owners	20 flocks of N. and W. L.	Free but in focus of infection	0	2044	0	2044	100
Bonifacio Perez	1. W. L.	Infected	1	22	0	22	100
Taytay, Rizal							
Muson Poultry	1. W. L.	Apparently free	0	442	0	442	100
Muson Poultry	2. W. L.	Infected	285	150	12	138	92

*Nc= Newcastle disease.

†N= Native. W.L.= White Leghorn. T.= Texas. M.= Minorca. Na.= Nagoya C.= Cantonese R.I.R.= Rhode Island Red.

perties, while potassium alum was used by Murae[12] to reduce the toxicity of, and enhance the antibody-forming properties of, dysentery vaccine. In a few trials, we found that chickens which were immunized with a vaccine (treated with 0.7% sodium borate and 0.1% formalin to which was added 0.5% potassium alum before its inoculation) were able to withstand challenge inoculations of 500 to 1,000 m.i.d.'s of virus, while the chickens given a vaccine similarly prepared but without potassium alum failed to withstand inoculations of the same amount of virus (500 to 1,000 m.i.d.'s) and developed the chronic form of the disease. This is a subject which will be discussed in a future paper.

Later reports from the field have shown the performance of vaccines prepared from duck embryos to be variable, and the protection derived to be of short duration (one to one and a half months) and not as dependable as that produced by vaccine of chicken embryo origin. In view of these findings, we discontinued the production of vaccines prepared from duck eggs.

Up to the termination of the preparation of this paper, more than 28,000 chickens and turkeys have been given the vaccine and not a single case of Newcastle disease due to a break of the vaccine had been reported. The extensive use of the vaccine has given very encouraging results. In all places where it has been tried, with the exception of four poultry farms where the infection was either too far advanced or where the duck embryo vaccine was exclusively used, it stopped the outbreaks. In spite of the poor performance in the field of the duck embryo vaccine, we consider that the extensive use of the vaccine in the field has been successful. This matter will be treated in our next paper.

SUMMARY AND CONCLUSIONS

The virus of Newcastle disease, or avian pest, was cultivated in 9-day-old chicken embryos in vast proportion. Passages of the cultivated virus did not seem to affect its virulence. We have observed that up to the fiftieth passage in chicken, duck, and turkey embryos, its virulence did not change.

A total of 55 different ways of vaccine preparation has been studied. The attenuating substances used were formalin, Merthiolate, phenol, tricresol, crystal violet, sodium ricinoleate, and sodium borate. Following the method of Mitchell, Walker, and Plummer[11] in the preparation of equine encephalomyelitis vaccine, a 10 per cent emulsion of the Newcastle disease infected chicken embryo in glycerinated phosphate buffer solution was made and treated with formalin. The preparation did not stimulate any protection among susceptible chickens. The same unfavorable results were experienced with similar preparations which were treated with the other attenuating chemicals herein mentioned. However, when we deviated from their method and utilized the amnionic fluid, the allantoic membrane, and the embryo proper emulsified in equal volume of glycerinated phosphate buffer solution and attenuated by adding 1 : 2,000 (0.05%) crystal violet and 0.05 per cent formalin, or 0.7 per cent sodium borate and 0.1 per cent formalin, and stored for ten days in an ordinary ice box, a protective vaccine was produced.

A preliminary statement could be made here that the addition of 0.5 per cent potassium alum to vaccines treated with 0.7 per cent sodium borate and 0.1 per cent formalin seems to enhance their antibody producing properties. In a few trials, the birds immunized with vaccines containing potassium alum were able to withstand inoculations of 500 to 1,000 m.i.d.'s of virus, while those given the vaccine without potassium alum were unable to do so.

A single dose of 1 cc. of this vaccine injected intramuscularly into the legs of chickens has proved protective in laboratory and field trials, as shown by the data included in this paper.

Laboratory trials as described in tables 3, 4, and 5 have shown that the vaccine

possesses a surprisingly high protection value — 100 per cent protection against 50 to 100 m.i.d.'s of virus.

Field trials of the product have given very promising results as shown in table 6. The value of the vaccine, like other vaccines, depends upon the status of the infection in the herd or flock prior to vaccination. If the vaccine is used early when there are only a few deaths in the flock, the percentage of protection is high, — 75 to 100 per cent as shown in table 6. The vaccine, however, was found to be of little value if administered when the great majority of the flock showed symptoms of the disease. The usefulness of the vaccine, therefore, is as a preventive. It is very doubtful if it has any curative value.

The keeping quality of the vaccine in refrigerator storage was found thus far to be three months. Further trials are being undertaken to determine whether the vaccine would keep beyond three months without affecting its potency.

More than 28,000 chickens and turkeys have been given the vaccine and the results thus far have been very encouraging. Not a single case of Newcastle disease due to a break of the product has been reported. The extensive vaccination in the field could have been more successful but for the variable performance of the duck embryo vaccine.

We believe that we have presented sufficient data to conclude that we have on hand a promising product which could be utilized in the control of Newcastle disease, or avian pest.

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Studies in Avian Leucosis. I. The Transmissibility of Visceral Lymphomatosis

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Review of the Literature

The earliest successful experimental transmission of avian leucosis was recorded by Ellermann and Bang in 1908[3]. Since that date many workers have confirmed their results (e. g. Hirschfeld and Jacoby*; Schmeisser[32]; Furth[13, 14, 15, 16]; Stubbs and Furth[33]; Furth and Breedis[39]; Engelbreth-Holm[9]; Jarmai[20]; Oberling and Guerin[41]; Olson[42, 46]; Uhl, Engelbreth-Holm, and Rothe Meyer[34]; Gibbs[31]; and Hamilton and Sawyer[28]. The transmissibility of at least the myeloid forms (erythroblastosis and granuloblastosis†) of this so-called disease complex has been well established. The combined results of all these experiments strongly suggest a close relationship if not an identity of erythroblastosis and granuloblastosis (and their etiology by a single filterable agent). The causative agent may occur as different strains which produce pure erythroblastosis in some cases, pure granuloblastosis in others, and in still others a mixture of both of these forms or an alternation of them in successive passages.

The etiology of the lymphogenous types of leucosis and their relationship to one another and to the myelogenous forms is not fully understood. Ellermann‡, Johnson,[20, 21, 22], and Patterson and Lee and their coworkers[10, 40, 18, 36, 37] reported that they produced several or all forms of leucosis in their transmission experiments and concluded that all types were different expressions of a single disease entity and were caused by a single agent. These conclusions are at variance with those of other workers in this field. It would appear that some of these authors may neither have sufficiently discounted the high natural incidence of all forms of leucosis and the extreme difficulty of obtaining stock which is relatively free of leucosis, nor have given sufficient consideration to the possibility that more than one causative agent may have been introduced in their experimental material. For example, Olson[41] pointed out that many of the cases of "lymphatic leukosis" described by Ellermann as occurring in his transmission experiments involving myelogenous material "were of spontaneous occurrence in chickens that had not been inoculated." The findings of some other investigators also failed to support a theory of the etiologic unity of myelogenous and lymphogenous types of leucosis. Furth[14] found 6 cases of lymphomatosis among 377 experimental birds inoculated with erythroblastic material. Since 2 cases were also observed among 193 untreated controls, he concluded that "lymphoid leucosis is as common in the control series as it is among the birds inoculated with material from myeloid or erythroleucosis." In summarizing, he said: "Evidence is wanting that lymphoid leucosis is caused by the agent that transmits myeloid and erythroleucosis. The occurrence of lymphoid leucosis among the birds inoculated with material from myeloid or erythroleucosis may be explained as spontaneous disease." Furth[14] also pointed out that "in mammals (guinea pig, mouse) lymphoid leucosis has been successfully transmitted; a change of type, however, from the lymphoid to the myeloid has never been observed." Similarly, in other successful transmission experiments with myelogenous strains, Stubbs and Furth[33] found 1 case each of lymphoid leucosis in inoculated and control birds, which they again interpreted as of spontaneous occurrence. Furth[15, 16] reported an avian leucosis strain (strain 2) which produced "lymphomatosis, myelomatosis, and endothelioma." However, Engelbreth-Holm[10] pointed out that "the cases of 'lymphomatosis' observed in Furth's strain 2 were characterized by large, immature cells, described by him as being 'like large lymphocytes (hemocytoblasts)', which differed distinctly from the relatively more differentiated small lympho-

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*Cited by Engelbreth-Holm[10].

†The nomenclature adopted in 1940 by the Committee of Pathologists meeting at the U. S. Regional Poultry Research Laboratory at East Lansing, Mich., is followed here.

‡Cited by Engelbreth-Holm[10].

cytes typical of the nontransmissible lymphogenous leukaemia. As Furth himself pointed out, the cell in question seems rather to suggest a nondifferentiated stem-cell." A careful reading of Furth's articles seems to confirm the probability of this interpretation of the cell type and suggests the conclusion that this so-called lymphomatosis is not a true lymphomatosis in the generally accepted meaning of the word, and that this strain, therefore, is not an example of myelogenous and lymphogenous forms of leucosis both being caused by a single agent. The same interpretation would apply equally to Furth's strain 12 ("mixed osteochondrosarcoma and lymphomatosis"[15]), which is a derivative of his strain 2. Furthermore, most investigators agreed, according to Engelbreth-Holm[10], that "among the enormous numbers of experimental birds observed, no more cases of lymphogenous leukaemia in its typical form have ever been noted [*i. e.*, among birds inoculated with myelogenous material] than might be found in any flock of fowls." Engelbreth-Holm and Rothe Meyer[11] reported that "among the total of 5,500 animals inoculated with one or another of our five strains we have not observed any case of transmissible lymphatic leukaemia [*i. e.*, lymphomatosis] but 3,100 cases of transmissible myelosis, haemocytoblastosis, and sarcoma. Nor have we succeeded in transmitting spontaneous lymphatic leukaemia to normal animals." Engelbreth-Holm[10] reported that he "did not observe even one case of lymphogenous leukaemia among approximately 7,000 birds inoculated wtih erythroleukaemic substance."

It will be noted that Engelbreth-Holm in the above quotation referred to "nontransmissible lymphogenous leukaemia." By this, he meant what is commonly referred to in this country as visceral lymphomatosis and what Furth[17] called "hepatolymphomatosis," differentiating it from the so-called lymphomatosis produced by his strain 2, as well as from neurolymphomatosis. Furth[17] also considered this visceral (or hepato-) lymphomatosis as so far nontransmissible, as did Olson[44], Andersen and Bang§ (who failed in ten different attempts to transmit it), Jarmai[29], Schaaf[51], and Hall, Bean, and Pollard[25, 26, 27]. Gibbs[21], however, reported the transmission of "lympholeukosis" (*i. e.*, visceral lymphomatosis) and concluded that "the causative agent in lympholeukosis is associated with a particular type of lymphoblast which may be distinguished from the lymphoblasts normally occurring in the blood by appropriate staining to bring out the arrangement of the chromatin material of the nucleus. This type of cell may be found in the liver, spleen, and portal blood of diseased chickens. Also, lympholeukosis may be transmitted to healthy susceptible chickens by inoculating portal blood containing large numbers of these living cells directly into the blood or abdominal cavity. If a virus is involved in lympholeukosis these experiments failed to indicate its presence."

The nature and transmissibility of lymphocytomata are also controversial. Jungherr[33, 34] considered them as expressions of neurolymphomatosis and, as such, transmissible with cell-containing materials. Fenstermacher[12] considered them as distinct from neurolymphomatosis and nontransmissible, while the latter is transmissible, although "both may co-exist in the same bird." Engelbreth-Holm[10] considered them as merely one form of "nontransmissible" lymphogenous leucosis. Olson[45], however, reported the transmission of a "lymphocytoma" through many passages; and Pentimalli[50] reported the successful transplantation of a "lymphosarcoma." These pathologic conditions would appear from the descriptions to be typical cases of visceral (or hepato-) lymphomatosis of a nodular type, in the former case affecting the liver, spleen, bursa of Fabricius, bone marrow, and ovary, and in the latter the liver, spleen, ovary, and kidneys. All these, with the exception of the bursa, are organs commonly affected in visceral lymphomatosis.

Of interest in this connection, as suggesting a possible parallel to the relationship between lymphocytomata and lymphosarcomata and visceral lymphomatosis, were the leucosis strains of Furth,[15, 16] Oberling and Guerin,[41] and Engelbreth-Holm and Rothe Meyer,[11] in which myelogenous forms of leucosis and sarcomata appeared side by side in successive passages of the same strains. Of their strain E-S, Engelbreth-Holm and Rothe Meyer[11] reported that "the method of inoculation has proved to be a very essential—and in many cases the decisive—factor in the outcome of the transmission of this strain: whether leukosis or sarcoma will develop in the inoculated animal....When blood from an animal attacked by leukosis from this strain is injected intravenously into normal animals, the takes will in most cases turn out to be pure leukosis. If, on the other hand, the same blood is injected intra-muscularly or subcutaneously, the takes will show in addition or exclusively a development of sarcoma at the site of inoc-

§Cited by Engelbreth-Holm[10].

ulation. When pieces of such a sarcoma are transplanted into normal animals the result will most often be development of sarcoma; a few of the animals will develop leukosis, besides sarcoma." Similarly, Uhl, Engelbreth-Holm, and Rothe Meyer[34] later succeeded in producing a diffuse leucosis with intravenous inoculation, and sarcomata with intramuscular or subcutaneous inoculation (using plasma alone in some experiments) of material from two strains of myelogenous leucosis. These strains (T and R) had previously produced only leucosis when inoculated intravenously for many more than 100 serial passages of each strain. Thus, they concluded that all types of disease in the same strain, whether leucosis or sarcoma, are produced by the same agent. On the other hand, Furth[16] found that when the agents of strain 1 (erythroblastosis) and of strain 11 (fibrosarcoma) were both inoculated into the same chicken they could later be separated again.

The relationship of neurolymphomatosis, or "fowl paralysis," to other forms of the so-called avian leucosis complex is likewise a matter of controversy. Johnson,[20, 21, 22] and Lee and Patterson and their coworkers,[26, 27, 43, 45, 49] as mentioned above, all considered neurolymphomatosis as merely one of several expressions of a single disease entity caused by a single agent and including all forms of leucosis. Hall and his coworkers[25, 26, 27] would eliminate visceral lymphomatosis from this group as nontransmissible, while considering neurolymphomatosis as but one expression of a complex including also the myelogenous forms of leucosis. Jungherr[24] recognized "erythrogranuloblastosis" and "neurovisceral lymphomatosis" as possibly two distinct entities caused by two different agents. On the other hand, Schaaf[31] questioned the possibility of a relationship of either "infectious" myelogenous leucosis (*i. e.*, erythroblastosis and granuloblastosis) or "non-infectious" lymphogenous leucosis (*i. e.*, visceral lymphomatosis) to "infectious fowl paralysis on etiological, hematological, pathologico-anatomical, histological, and epizoötiological grounds." On this point, Engelbreth-Holm[10] concluded that "until further information is available it is hardly possible to decide whether neurolymphomatosis gallinarum is a disease fundamentally different from all leukaemias, or whether neurolymphomatosis and the so-called lymphogenous fowl leukaemia [*i. e.*, visceral lymphomatosis] are to be included in one group that is different from myelogenous leukaemia and erythroleukaemia [*i. e.*, granuloblastosis and erythroblastosis]."

The transmissibility of both ocular and neural types of lymphomatosis has been fairly well established. Although Barber[1], Olson[44], and Beach[2] ‖ reported failure in transmission attempts, Pappenheimer, Dunn, and Seidlin[47], Furth[15, 16], Gibbs[21], Gildow, Williams, and Lampman[22, 23], Jungherr[23], Durant and McDougle[7], Blakemore[3], Glover[24], Brandly, Nelson, and Cottral[4], and Hall, Bean, and Pollard[25] were all successful in the transmission of neurolymphomatosis by various means. Except in the case of Hall, Bean, and Pollard[25] (who apparently selected for use in later serial passages a strain of erythroblastosis occurring spontaneously, or adventitiously introduced with the original neurolymphomatotic material), and the case of Brandly, Nelson, and Cottral[4] (who found osteopetrosis in 9 per cent of inoculated birds in a succession of serial passages initiated with material originating from cases of neurolymphomatosis), transmission of neurolymphomatosis generally occurred uncomplicated by the appearance of more cases of other forms of leucosis among the experimental birds than can be explained satisfactorily as spontaneous. On the other hand, the transmission experiments with osteopetrosis reported by Jungherr and Landauer[25] seemed to produce neurolymphomatosis, visceral lymphomatosis, and osteopetrosis indiscriminately with a single strain.

Thus, excepting neurolymphomatosis (and possibly ostepetrosis), the only recorded transmission experiments showing transmission of lymphomatosis *as such* seem to be those of Gibbs's[21] "lympholeukosis," of Burmester's and Prickett's[5] and possibly Olson's transmissible "lymphocytoma"[45] and Pentimalli's transmissible "lymphosarcoma"[39], all of which probably represent the true visceral lymphomatosis heretofore widely considered nontransmissible.

In an attempt to throw further light on this involved subject, a series of transmission experiments with visceral lymphomatosis was initiated in 1943, the first of which is reported here.

OBJECTIVES

The primary objective of this experiment was to attempt to transmit visceral lymphomatosis, a form of the avian leuco-

‖Cole[6], however, says that statistical anaylsis of Beach's results shows that transmission was actually accomplished.

sis complex heretofore widely considered nontransmissible, by the inoculation of lymphomatous material from a field case.

Secondary objectives were (1) to determine whether the causative agent is concentrated in any particular organ or organs; (2) to obtain further information on a possible relationship of sex hormones to visceral lymphomatosis by the study of the effects of caponization on the incidence of the disease; and (3) to investigate the possibility of increasing the virulence of the causative agent and shortening the incubation period for the disease by serial passage through young chicks.

EXPERIMENTAL PROCEDURE

In the main transmission experiment, 425 chickens were used, and 84 additional chicks were used in serial passages, a total of 509 birds.

On Dec. 22, 1943, 301 White Plymouth Rock chicks 40 days of age were inoculated with leucotic organs from a field case of typical visceral lymphomatosis in a Barred Plymouth Rock hen. Macroscopically, this bird showed great enlargement of the liver, spleen, ovary, and kidneys. (Samples of all of these organs, and the heart as well, were preserved in neutralized 10 per cent formalin and prepared for microscopic study.) Histologically, the liver, spleen, ovary, and kidneys all showed a marked diffuse infiltration of lymphoblasts.

The organs and blood of the donor were collected separately under aseptic conditions. The organs were triturated individually in sterile physiologic saline solution, while the blood was defibrinated and diluted with sterile saline solution. The chicks were inoculated intraperitoneally with 3 cc. each of these suspensions of lymphomatous material in the following groups: 50 each with liver, heart, spleen, ovary, and defibrinated blood; and 51 with kidney. As controls, 124 chicks from the same source and of the same age were kept untreated but under similar conditions.

Eight weeks after inoculation, liver biopsy operations were begun on a random sample of 12 chickens from each of the six inoculated groups, or a total of 72 inoculated birds, and on 24 controls. These operations were continued on the same chickens at monthly intervals throughout the entire course of the experiment or until the death of the chickens. The technique used and the results obtained will be described in another paper.

Five weeks after inoculation, 182 males from both the inoculated and the control groups were caponized, both to study the effects of caponization on the incidence of the disease and to avoid the necessity of disposing of the males at broiler age.

In addition to the main transmission experiment, three serial passages through small groups of chicks were made in an attempt to increase the virulence of the causative agent and to shorten the incubation period.

In serial passage 2, 20, 14-day-old chicks were inoculated intraperitoneally on March 27, 1944, with 3 cc. each of a trituration of liver and spleen from a typical case of visceral lymphomatosis which appeared among the inoculated birds in the original transmission experiment. At the same time, 2 mm. cubes of a pectoral lymphoblastoma from another case of visceral lymphomatosis among the inoculated birds were transplanted in the pectoral muscles of 5 other chicks. Eighteen chicks of the same lot were kept as controls.

On April 4, 1944, in serial passage 3, 10, 13-day-old chicks were inoculated intraperitoneally with 3 cc. each of a trituration of liver in normal saline solution. The donor for this third serial passage was a case of visceral lymphomatosis which developed in 1 of the chicks in serial passage 2, which had received the tumor transplant in the pectoral muscle. Ten chicks of the same lot as those inoculated with this material were kept untreated as controls.

In serial passage 4 on May 8, 1944, 11, 27-day-old chicks were inoculated intraperitoneally with 3 cc. each of a trituration of liver in normal saline solution from a case of visceral lymphomatosis which developed among the inoculated chicks in serial passage 3. Nine chicks of the same lot served as controls.

Meanwhile, and during the entire course of the experiment until the survivors were killed and examined between Jan. 12, 1945, and Feb. 1, 1945, at the age of 428 to 448 days (or 388 to 408 days after inoculation), autopsies were performed on all birds which died. Detailed autopsy observations were recorded, and tissues were saved for histologic study from most of the definitely lymphomatotic, and all of the doubtful, cases. Samples of liver, heart, spleen, kidney, and sometimes ovary, of 113 cases or suspected cases in the main transmission experiment, and 36 cases from the serial passage groups, were pre-

served in neutralized 10 per cent formalin, embedded in paraffin, sectioned, stained with hematoxylin and phloxine, and studied during the course of the experiment.

RESULTS

Results of Transmission.—The results of the main transmission experiment are summarized in table 1. Of 301 birds inoculated with lymphomatous material, 117 (38.8 %) developed visceral lymphomatosis,

TABLE 1—Incidence of Visceral Lymphomatosis in Various Groups by Inoculum

Inoculum	No. in group	Pos. cases	% of group	Av. age at death (days)*
Liver	50	24	48.0	184
Kidney	51	24	47.1	212
Spleen	50	22	44.0	204
Blood	50	20	40.0	232
Ovary	50	15	30.0	215
Heart	50	12	24.0	269
Total	301	117	38.8	214
Controls	124	12	9.6	293

*Birds which died of hemorrhage during caponization and showed early lymphomatosis are not included here.

Pos.=positive.

while only 12 (9.6 %) of the controls developed the disease, a number well within the range of the average spontaneous incidence of visceral lymphomatosis in the field. In other words, the incidence increased slightly more than 300 per cent in the inoculated birds over the controls.

At the same time, 4 cases of neurolymphomatosis (1.3 %) appeared among the inoculated birds, and 2 cases (1.6 %) among the controls. Three cases of granuloblastosis were found in the inoculated group, an incidence of 0.9 per cent, compared with 2.4 per cent (or 3 cases) in the control birds. No cases of erythroblastosis or osteopetrosis were found in either inoculated or control groups. In interpreting these results, the possibility of using preinfected stock must be borne in mind. Thus, to summarize, *no type of leucosis other than the visceral lymphomatosis which was inoculated was increased in the inoculated group as compared with the control group.*

The average age at death in cases of visceral lymphomatosis was 214 days (or 174 days after inoculation with lymphomatous material) in the inoculated birds, compared with an average of 293 days in the spontaneous cases appearing among the control birds (*see* table 1).

The results of the inoculation of individual organs into various groups of birds in the inoculated flock showed 24 cases of visceral lymphomatosis (48%) in the liver-inoculated group, which died in an average of one hundred forty-four (144) days after inoculation at a mean age of 184 days; 24 cases (47.1 %) in the kidney-inoculated group, which died in an average of one hundred seventy-two (172) days after inoculation at a mean age of 212 days; 22 cases (44%) in the spleen-inoculated group, which died in an average of one hundred sixty-four (164) days after inoculation at a mean age of 204 days; 20 (40%) in the blood-inoculated group, which died in an average of one hundred ninety-two (192) days after inoculation at a mean age of 232 days; 15 (30%) in the ovary-inoculated group, which died in an average of one hundred seventy-five (175) days after inoculation, or at a mean age of 215 days; and 12 (24%) in the heart-inoculated group, which died in an average of two hundred twenty-nine (229) days after inoculation, or at a mean age of 269 days.

Results of Caponization.—In the inoculated birds, the incidence of visceral lymphomatosis among females was found to be 54 cases (38.5 %) among the 140 inoculated females included in the experiment; 48 cases (53.9 %) among the 89 capons included; none in the 17 slips included; 13 cases (24.5 %) among the 53 males included among the inoculated birds; and 13 cases (18.5 %) among the 70 males and slips together.

In control birds, the incidence among females was found to be 8 cases (13.3 %) among 60 females; 3 cases (11.1%) among 27 capons; none among the 21 slips; 1 case (6.2%) among 16 males included in the control group; and 1 case (2.7%) in a total of 37 males and slips. Of 2 ovariectomized females, both died of visceral lymphomatosis, but the group was too

small to have any statistical significance and is not included in the above percentages for that reason. Several males which died of hemorrhage as a result of the caponizing operation, and which showed early lymphomatosis on autopsy (either histologically, or both grossly and histologically) were included with the normal males. These results are summarized in table 2.

TABLE 2—Results of Castration on the Incidence of Visceral Lymphomatosis

	Total No.	Pos. cases	% of group
Inoculated birds			
Females	140	54	38.5
Ovariectomized	2	2	100.0
Capons	89	48	53.9
Slips	17	0	0.0
Males	53	13	24.5
Males & slips	70	13	18.5
Controls			
Females	60	8	13.3
Capons	27	3	11.1
Slips	21	0	0.0
Males	16	1	6.2
Males & slips	37	1	2.7

Results of Serial Passage.—Although the groups of chicks used for serial passage were too small to have much statistical significance, the results obtained are summarized in table 3. Further work involving larger groups of chicks is now in progress.

In serial passage 2, 13 of 20 (65%) of the inoculated birds died of visceral lymphomatosis at an average age of 64 days, or an average of fifty days after inoculation; 2 of 5 (40%) of the birds which received tumor transplants in the pectoral muscle died of visceral lymphomatosis at an average age of 65 days, or in an average of fifty-one days after tumor transplantation; and 2 of 18 (11.1%) control birds died of visceral lymphomatosis at an average age of 130 days.

In serial passage 3, 9 (90%) of the 10 inoculated birds died of visceral lymphomatosis at an average age of 37 days, or in an average of twenty-four days after inoculation, while none of the controls contracted the disease.

In serial passage 4, 9 (81.8%) of the 11 inoculated birds died of visceral lymphomatosis at an average age of 56 days, or in an average time of twenty-four days after inoculation, while none of the controls developed the disease.

No cases of neurolymphomatosis, osteopetrosis, granuloblastosis, or erythroblastosis appeared in any of the serial passage groups, either inoculated or control.

Histologic Results.—Samples of liver, heart, spleen, kidney, and in some cases ovary from 150 positive or suspected cases of visceral lymphomatosis were studied microscopically, including tissues from 14 controls and 99 inoculated birds in the main transmission experiment, and 35 inoculated birds and 2 controls from the serial passage groups.

Of the 14 controls from the main experiment studied histologically, 2 showed no microscopic evidences of leucosis; 3 showed granuloblastosis, with marked myeloblastic infiltration of the liver, spleen, and kidneys in all 3, and the heart in 2. The remaining 9 showed visceral lymphomatosis, with lymphoblastic or lymphocytic infiltration of the organs, involving the liver in all 9 cases, the spleen in 7, and the heart and kidneys each in 6, while the ovary was infiltrated in all 4 of the 9 lymphomatotic cases in which it was examined.

Among the 99 inoculated birds from the main experiment which were studied histologically, 16 proved microscopically negative for leucosis; 2 showed granuloblastosis; 1 showed a mixture of both granuloblastosis and lymphomatosis; and 80 showed visceral lymphomatosis. One of the 2 cases of granuloblastosis showed the liver, spleen, kidneys, and heart all markedly infiltrated with myeloblasts, and the other showed the kidneys to be markedly infiltrated with myeloblasts, as well as a large myeloblastoma of the right pectoral muscle. The case of mixed granuloblastosis and visceral lymphomatosis showed numerous areas of both lymphoblasts and myeloblasts in the liver, spleen, heart, kidneys, and ovary, with lymphoblastic areas predominating in the liver, spleen, and heart, and myeloblastic areas in the kidneys and ovary. Of the 80 purely lymphomatotic

cases, 68 showed lymphoid infiltration of the liver; 65 of the spleen; 64 of the kidneys; 55 of the heart; and 14 (of the 18 in which the ovaries were included in the microscopic examination) infiltration of the ovary (*see* table 4).

TABLE 3—Incidence of Visceral Lymphomatosis in Serial Passage Groups of Chicks

Group	No. of chicks	Pos. cases	% of group	Age at death (days)	Death days after inoc.
S P 2 Inoc.	20	13	65.0	64	50
S P 2 Trans.	5	2	40.0	65	51
S P 2 Con.	18	2	11.1	130	116
S P 3 Inoc.	10	9	90.0	32	24
S P 3 Con.	10	0	0.0	..	..
S P 4 Inoc.	11	9	81.8	56	29
S P 4 Con.	10	0	0.0	..	..

S P = serial passage. Inoc. = inoculated. Trans. = transplants. Con. = controls. Pos. = positive.

Thus, among the 90 cases in the main experiment which proved to be microscopically positive for visceral lymphomatosis, including both inoculated and control birds, 77 (85.5%) showed liver lesions; 72 (80%) showed spleen lesions; 70 (77.7%) showed kidney lesions; 61 (67.7%) showed heart lesions; and 18 of the 22 cases in which the ovary was studied (81%) showed ovarian lesions.

Lymphoblasts were the predominating cell type of the lymphomatotic lesions, but lymphocytes were the typical cells in 1 case where the most conspicuous lesion was a large lymphocytoma of the ovary accompanied by less marked, diffuse, lymphocytic infiltration of the liver and spleen.

Of the 2 controls from serial passage 2 which were studied histologically, both showed visceral lymphomatosis, with lymphomatotic infiltration of the liver, spleen, kidneys, and heart in both cases, and of the ovary in the 1 case in which that organ was studied microscopically. Of 17 inoculated birds examined from serial passage 2, 15 proved to be positive for visceral lymphomatosis, as did the 9 each examined from the inoculated birds of serial passages 3 and 4. Of these 33 positive cases from three serial passages, 31 (93.9%) showed heart lesions; 31 (93.9%) showed kidney lesions; 30 (90.9%) showed liver lesions, 30 (90.9%) showed spleen lesions, and the 8 cases in which the ovary was studied all showed ovarian lesions.

An interesting observation concerning the specificity (or lack of specificity) in lymphoid cell types in transmission was made during the serial passages subsidiary to the main transmission experiment. One inoculated bird in the main transmission experiment which developed marked lymphoblastic infiltration of the liver and spleen, as well as a large lymphoblastoma of the pectoral muscle, was used as a donor for tumor transplantation in serial passage 2. Fresh pieces of the lymphoblastoma were transplanted into the pectoral muscle of 5, 14-day-old chicks. Two of these developed a general lymphomatosis, 1 in 28 days, the other in 74 days after the transplantation. In the former, the invading cell was a lymphoblast which infiltrated

TABLE 4—Distribution of Microscopic Lesions in Cases of Visceral Lymphomatosis in the Main Transmission Experiment

Group by inoculum	No. pos. cases	No. of cases with lesions in: Liver	Spleen	Kidney	Heart	Ovary
Liver	20	16	14	14	15	4*
Kidney	17	13	14	12	15	4†
Spleen	16	16	14	14	8	1‡
Blood	13	11	8	11	7	3§
Ovary	8	6	8	6	6	2*
Heart	7	6	7	4	7	0‖
Total	81	68	65	64	55	14**
Controls	9	9	7	6	6	4*

* Of 4 examined. † Of 5 examined. ‡ Of 2 examined. § Of 3 examined. ‖ None examined. ** Of 18 examined.

the liver, spleen, kidneys, and particularly the heart; in the latter, the liver, spleen, heart, and ovary were extensively infiltrated with lymphocytes. The lymphoblastic case was used as a donor for the third serial passage, which in turn was followed by a fourth with the donor selected at random from cases occurring in the third serial passage group. In some of the cases in both serial passages 3 and 4, the typical cell of the infiltrated areas was a lymphoblast while, in others, it was a more mature lymphocytic cell, suggesting a lack of spec-

ificity in the type of lymphoid cell (*i. e.*, with respect to maturity) which may be affected by at least this particular strain of visceral lymphomatosis. Interestingly enough, this strain, which apparently showed an affinity for muscle in the donor selected from the main transmission experiment, produced particularly extensive infiltration of the heart muscle in all but 1 of the 18 cases appearing in the third and fourth serial passages.

DISCUSSION

The fact that no type of leucosis, other than the visceral lymphomatosis which was inoculated, was increased in the inoculated group as compared with the control group, and the fact that the incidence of other types of leucosis in the control group was no higher than the average for spontaneous cases of these types of leucosis in the field, suggest that such cases of both neurolymphomatosis and granuloblastosis that did occur in both inoculated and control groups should be considered as spontaneous, and that visceral lymphomatosis was transmitted in this experiment as a separate entity distinct from both neurolymphomatosis and osteopetrosis and from the myelogenous forms of avian leucosis, erythroblastosis and granuloblastosis.

The variation in the percentage of takes found in the several groups of birds inoculated with different lymphomatous organs—between 24 per cent in the heart-inoculated group and 48 per cent in the liver-inoculated group, with the other groups intermediate—would not seem to be great enough, except possibly in the extremes, to show a marked concentration of the causative agent in any of the organs used. A comparison with the results of a similar experiment conducted by this laboratory in 1942 and 1943, and published in the Fourth Annual Report of the U. S. Regional Poultry Research Laboratory at East Lansing, Mich.,[15] is interesting. In that experiment, 25 per cent of the kidney-inoculated birds, 22.5 per cent each of the spleen- and blood-inoculated, 10 per cent of the liver-inoculated, 7.5 per cent of the ovary-inoculated, and 2.5 per cent of the heart-inoculated birds died of visceral lymphomatosis, compared with 7.5 per cent of the uninoculated controls. Repetition of this phase of the experiment will show whether such differences as were found will appear with any consistency in repeated trials.

The incidence of visceral lymphomatosis was found to be greater in females than in males, or than in males and slips together, in both inoculated and control groups. Caponized males showed a decidedly higher incidence than either normal males or slips or females in the inoculated group while, in the control group, the capons showed an incidence slightly lower than that among females, but markedly higher than that in normal males or slips. It should be borne in mind that the males in the main transmission experiment were caponized five weeks after the chickens had been inoculated with lymphomatous material. The data indicate that the capons were more susceptible than the normal males or slips, and this, in turn, suggests that the androgenic hormones (their absence, in this case) are somehow related to susceptibility or resistance to visceral lymphomatosis. If caponization had been performed prior to inoculation, it is entirely possible that a still greater incidence of lymphomatosis would have occurred. Experiments to test the validity of this hypothesis are contemplated.

Although the number of chicks used for the exploratory serial passage of visceral lymphomatosis reported here was too small to be definitive, the results suggest that the virulence of the causative agent apparently can be increased, and the incubation period shortened, by serial passage through young chicks. The slightly longer incubation period for the disease in serial passage 4 than in serial passage 3 was possibly related to the greater age at inoculation and, therefore, possibly decreased susceptibility, of the chicks used in the later passage.

The histologic study of cases of visceral lymphomatosis suggests that lesions occur with relatively equal frequency in the organs studied—liver, spleen, kidney, heart, and ovary. Although the lymphoblast is predominantly the typical cell of the lymphomatous lesion, cases in which a more mature lymphocyte is the predominating cell apparently may occur indiscriminately within a single strain of visceral lymphomatosis.

SUMMARY AND CONCLUSIONS

1) The incidence of visceral lymphomatosis was apparently increased, by inoculation with cell-containing material from a lymphomatous field case, from an incidence of 9.6 per cent in controls to an incidence of 38.8 per cent in inoculated birds.

2) Visceral lymphomatosis was transmitted apparently as a separate entity distinct from other forms of avian leucosis, since no increase in the incidence of any type of leucosis (other than the visceral lymphomatosis which was inoculated) was found in the inoculated birds as compared with the controls.

3) By inoculation with lymphomatous material, the incubation period for visceral lymphomatosis was apparently shortened, *i. e.*, the controls which succumbed to disease died at an average age of 293 days while the inoculated birds which succumbed to disease died at an average age of 214 days.

4) The results of the inoculation of liver, kidney, spleen, ovary, heart, and defibrinated blood from a lymphomatotic field case in several groups of inoculated birds (*i. e.*, one organ to each group) showed that deaths from visceral lymphomatosis in the several groups varied between 24 per cent in the heart-inoculated group to 48 per cent in the liver-inoculated group, with the other groups intermediate, suggesting that there is no marked concentration of the causative agent or absence of it in any certain organ or organs.

5) Females appeared to be more susceptible to visceral lymphomatosis than males or slips; and capons appeared more susceptible than males or slips, and at least as susceptible as females.

6) The incidence of visceral lymphomatosis can apparently be increased and the average incubation period for the disease simultaneously shortened by serial passage through young chicks.

7) In cases of visceral lymphomatosis, histologic lesions were found in 85.5 per cent of the livers examined, in 81 per cent of the ovaries, 80 per cent of the spleens, 77.7 per cent of the kidneys, and 67.7 per cent of the hearts in the main transmission experiment; and in 100 per cent of the ovaries examined, 93.9 per cent of the kidneys and hearts, and 90.9 per cent of the livers and spleens in the serial passage cases.

8) In the strain of visceral lymphomatosis studied, the typical cell found in the lymphomatous lesions of positive cases varied in maturity from lymphoblasts to lymphocytes, with lymphoblasts greatly predominating.

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Studies in Avian Leucosis. II. The Use of Biopsy Technique in the Study of Visceral Lymphomatosis

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Review of the Literature

Although liver biopsy has been used successfully for many years as a diagnostic technique in human medicine (Iversen and Roholm[4], Tripoli and Fader[5, 9]), and recently has been reported in use with sheep (Dick[1]), this technique has not been used, generally, in the study of avian pathology. Marine and Rosen[6] mention that in the study of the effect of caponization on the incidence of lymphomatosis in fowls "routine liver biopsies have shown that this organ may be transformed from the normal (about 40 g), reddish brown color to a huge (300 g), reddish gray organ in fifteen days," but no further details are given concerning the technique used.

In February, 1944, a series of liver biopsy operations on chickens in a transmission experiment on visceral lymphomatosis[11] was undertaken to investigate the possibilities of liver biopsy in the study of avian leucosis.

Objectives

The primary objective of the liver biopsy operations was to study the histopathologic changes over a period of months in the livers of chickens inoculated with visceral lymphomatosis as compared with the livers of controls. In particular, an attempt was made to gain some information on the length of the incubation period for this disease, the possibility of recovery in affected birds, and the degree of lymphoid accumulation in the liver which may be considered normal. An attempt also was made to determine the feasibility of a practical application of liver biopsy technique to the control of visceral lymphomatosis by the detection through this means of early cases of the disease which might act as carriers.

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Experimental Procedure

Ten weeks after 301, 40-day-old chickens in a transmission experiment had been inoculated with lymphomatotic organs from a field case of visceral lymphomatosis, monthly liver biopsies were initiated on a series of 96 chickens, including 72 inoculated birds and 24 controls. These were divided into four groups of 24 each, including 18 inoculated birds and 6 controls, which were rotated weekly with each group being operated on at monthly intervals. These are the four groups for which results are tabulated in tables 1, 2, 3, and 4. A few additional biopsies were performed at two-week intervals on birds which the first biopsy had shown to be of particular interest, but such biweekly operations were not continued because of the difficulties attending such frequent surgery. In September, after the fifth biopsy but before the sixth, all of the healthy males surviving in the transmission experiment were sold, including a few of those in the biopsy series. At this time, the chickens were 10 months old and had been inoculated nearly nine months previously. A total of 457 liver biopsies were performed over a period of nearly ten months, with some of the birds surviving to be operated on for as many as nine times each.

During the operations, three sets of instruments and needles were used. These were rotated and between operations were kept in jars of 70 per cent alcohol in order to avoid as much as possible the transfer of infective material from 1 chicken to another. For the same reason, the control chickens were always operated on first.

For operating, the chickens were fastened to a caponizing bow. An incision about 1 inch long was made through the body wall just posterior to the last rib, and more ventrally than for a caponizing operation. A piece of liver about the size of a garden pea was then pinched out from the exposed liver lobe with a Gaylor uterine specimen forceps. In closing the incision, one stitch was placed in the body wall with catgut threaded in a curved needle and another stitch in the skin. To avoid interference by adhesions and scar

tissue, which developed in many cases, the first two operations were performed on the left side, the third and fourth on the right, the fifth and sixth again on the left, and so on. In the few cases where hernias resulted, the condition was corrected in the succeeding operation.

Death losses resulting from the operations were low. In the first operation particularly, losses were no greater than might be expected from caponizing operations. As additional operations were performed on the same birds, the mortality became somewhat higher. In the course of the biopsies described here, only 1 bird was lost from the 96 in the group as result of the first operation. Three each died after the second and third operations, 4 following the fourth, and 9 out of the remaining 53 birds after the fifth operation. Thereafter, in the succeeding operations, losses continued to increase until only 12 of the original 96 birds survived for the ninth and last operation of the series. These figures suggest that four operations is the maximum that can be performed on the same bird with reasonable safety. Most of the deaths resulted from hemorrhage which, in turn, generally resulted from the breakdown of adhesions caused by previous operations. Many of the birds which died showed developing lymphomatosis, and this condition may have increased the tendency to hemorrhage. If biopsy technique should ever become practical for diagnostic purposes, only one operation would be needed for each bird, and this should not be dangerous.

Since previous experimentation had shown Bouin's fixative, one of several tried, to be the most satisfactory preservative for chicken tissues the liver tissues collected by biopsy were preserved in this fixative for twenty-four to forty-eight hours. The butyl alcohol method of dehydration was used, including the following steps:

35% ethyl alcohol — 1 to 2 hours.
50% ethyl alcohol — 1 to 2 hours.
70% ethyl alcohol — several changes, totalling at least 24 hours.
2/3 70% ethyl alcohol + 1/3 normal butyl alcohol — 1 to 2 hours.

TABLE 1—Results of Liver Biopsies—Group I

Chicken no. and status	Op. 1 Date 2-16	2 3-24	3 4-21	4 5-19	5 6-30	6 9-8	7 10-13	8 11-15	9 12-13	Date of death or sale
					Microscopic findings					
16—I	***o	l	..	..	..	..	..	..	..	3-27‡
41—I	**o	lee	le	..	..	..	..	..	..	5-8‡
49—I	*o	**o	**o	*o	*o	..	..	..	..	7-5†
58—I	***o	***f	**e	***e	***f	**f	**	**	***	12-13K‡
82—I	*o	**e	**f	**f	lef	***f	***f	l	..	11-15‡
96—I	*o	**o	**f	*o	of	..	..	..	..	7-5†
102—I	**e	**o	**e	**f	**e	..	..	..	..	7-5†
128—I	***o	**o	**o	**o	***o	..	..	..	..	7-19‡
146—I	*o	**o	**e	***o	lf	..	..	..	..	7-18‡
160—I	**o	**o	**f	**e	***e	..	..	..	..	7-5‡
185—I	**o	l	***ee	***ee	***eef	..	..	..	..	7-30†
193—I	**o	*o	**e	*f	*e	*f	**	..	..	11-3†
221—I	*o	*e	*f	**o	..	..	..	..	..	5-24†
224—I	*o	*o	*o	**f	**o	..	..	..	..	7-7‡
248—I	*o	**o	**o	**f	..	..	..	..	..	5-24†
262—I	**o	**o	**o	**o	**o	**f	**f	***	***e	12-13K†
287—I	*o	*o	*o	*f	*o	**f	**	*o	**o	12-13K†
293—I	*o	..	..	..	..	..	..	..	..	3-6†
312—C	*o	*o	**f	*o	..	..	..	..	..	6-12†
338—C	*o	**o	*e	*e	**o	..	..	..	..	9-6S†
372—C	**o	**o	**o	**e	*e	**f	**o	**o	*o	12-13K†
376—C	**o	**f	*o	..	..	..	..	..	..	4-21†
394—C	*o	*f	*f	..	..	..	..	..	..	4-24†
396—C	*o	*f	*f	*o	*f	*o	..	..	..	10-7†

o=No lymphoid accumulations present. *=Small lymphoid accumulations present. **=Moderate lymphoid accumulations present. ***=Large lymphoid accumulations present. l=Lymphomatosis. e=Eosinophils present in lymphoid areas. ee=Numerous eosinophils present in lymphoid areas. f=Liver very fatty. K=Killed on date given at termination of experiment. S=Male sold on date given; apparently normal when sold. I=Inoculated chicken. C=Control chicken. Op.=Operation. †=Absence of visceral lymphomatosis *post mortem*. ‡=Presence of visceral lymphomatosis *post mortem*.

1/3 95% ethyl alcohol + 2/3 normal butyl alcohol — 1 to 2 hours.
Absolute normal butyl alcohol — 2 hours.
Fresh, absolute normal butyl alcohol — overnight.
3 changes of paraffin — 2 hours each.
Embed in fresh paraffin.

The tissues were sectioned and stained routinely with Mayer's hematoxylin and phloxine. In some cases, duplicate slides were also stained with Harris's hematoxylin and orange G for comparison.

RESULTS

The results of the biopsy operations do not lend themselves readily to summary. They are, therefore, presented in tabular form in tables 1, 2, 3, and 4 with an accompanying key.

Of the 96 chickens operated, only 2 showed a complete absence of perivascular lymphoid accumulations in the first biopsy specimen taken from the liver. Of these 2, both of which were birds that had been inoculated with lymphomatotic material, 1 died of visceral lymphomatosis within a month, showing lymphoblastic accumulations microscopically in the liver, spleen, heart, and ovary at death. The other showed moderate perivascular lymphoid accumulations in the second and third biopsy specimens, but died shortly thereafter of intercurrent disease, showing no gross or microscopic evidence of lymphomatosis at that time. All other birds studied, controls as well as inoculated, showed some lymphoid accumulations, usually perivascular, in every biopsy liver sample taken, with three exceptions. In this connection, it is interesting to note that in a histologic study of the livers of lymphomatosis-free stock Waters and Prickett[10] found that

TABLE 2—Results of Liver Biopsies—Group 2

Chicken no. and status	Op. 1	2	3	4	5	6	7	8	9	Date of death or sale
	Date 3-3	3-31	4-28	5-26	7-7	9-8	10-13	11-15	12-13	
	Microscopic findings									
1—I	o	..	..	..	..	..	..	..	..	3-27‡
42—I	**	***e	**f	**e	*	..	..	..	..	9-6S†
45—I	**	..	..	..	..	..	..	..	..	4-4‡
65—I	**	**e	***eef	**eef	**	..	..	..	..	9-6S†
69—I	*	**	..	..	..	..	..	..	..	4-10†
98—I	**	**	**	**ef	*	*f	**	*f	*	12-13K†
107—I	**	**e	**e	*	..	..	..	..	..	6-3†
115—I	**e	..	..	..	..	..	..	..	..	3-17‡
123—I	**	*	..	..	..	..	..	..	..	4-8‡
161—I	**	**	..	..	..	..	..	..	..	4-3‡
166—I	*	**	**f	**	..	..	..	..	..	5-29†
191—I	***	**	**ef	***f	***	***	le	..	..	11-15‡
213—I	***	..	..	..	..	..	..	..	..	3-9†
222—I	**	***	**f	**f	**	..	..	..	..	7-7‡
229—I	*	***	*f	**eef	**	..	..	..	..	9-6S†
260—I	**f	lf	lf	lf	l	..	..	..	..	7-7‡
272—I	*	**	*	**f	**	..	..	..	..	7-7‡
274—I	**e	***e	le	l	lf	lf	le	..	..	11-6‡
302—C	*	*ef	*e	**eef	*f	**	***	**	l	12-13K‡
318—C	*e	**	**ef	*e	*	..	..	..	..	8-8†
335—C	*	**f	*	*f	*	**f	***	..	..	10-16‡
343—C	*	**	**	**e	**e	..	..	..	..	9-6S†
345—C	*	**f	**	**	..	..	..	..	..	8-7†
369—C	**eef	**	**e	*	..	..	..	..	..	5-7†

o=No lymphoid accumulations present. *=Small lymphoid accumulations present. **=Moderate lymphoid accumulations present. ***=Large lymphoid accumulations present. l=Lymphomatosis. e=Eosinophils present in lymphoid areas. ee=Numerous eosinophils present in lymphoid areas. f=Liver very fatty. K=Killed on date given at termination of experiment. S=Male sold on date given; apparently normal when sold. I=Inoculated chicken. C=Control chicken. Op.=Operation. †=Absence of visceral lymphomatosis *post mortem*. ‡=Presence of visceral lymphomatosis *post mortem*.

"numerous specimens were seen . . . where no perivascular accumulations were to be found while an adjacent section, from the same block, showed numbers of perivascular nodules." Three chickens, 2 controls and 1 inoculated bird, each had one liver sample entirely free of lymphoid accumulations, although the preceding and succeeding samples in all 3 of these all showed small or moderate-sized lymphoid areas. None of these 3 cases developed lymphomatosis. The lymphoid areas thus commonly found throughout the series of liver samples generally formed well-defined nodules without outward diffusion. When such diffusion occurred, it was generally in large lymphoid areas and in cases where lymphomatosis was apparently developing or definitely present.

Among the 96 birds studied, 21 of the 42 which developed visceral lymphomatosis showed, in general, a progressive increase in the size and number of lymphoid areas in the liver over a period of several months. Ten other of these 42 birds showed no marked change in the amount of lymphoid tissue in the liver during the several months preceding death from visceral lymphomatosis. The remaining 11 positive cases died after only one or two biopsies had been performed, and thus could not be observed over an extended period. Many of the two latter groups, which showed no marked progressive increase of lymphoid tissue in the liver for more than a month preceding death from visceral lymphomatosis, showed no greater accumulation of lymphoid tissue in the liver a month or less previous

TABLE 3—Results of Liver Biopsies—Group 3

Chicken no. and status	Op. 1 Date 3-10	2 4-7	3 5-5	4 6-2	5 7-14	6 9-15	7 10-13	8 11-15	9 12-13	Date of death or sale
				Microscopic findings						
27—I	**	**e	***e	**	..	..	..	..	..	6-12‡
33—I	l	l	..	..	..	..	..	..	..	4-17‡
48—1	**	**	**e	**	**f	***	**	..	..	10-16‡
55—I	**	lef	**ee	**ee	**eef	..	..	..	..	9-6S†
94—I	**	*f	*	*f	*f	*f	*f	**	*	12-13K†
97—I	**	**ef	..	..	..	..	..	..	..	4-15†
105—I	**	***	..	..	..	..	..	..	..	4-14‡
112—I	***	..	..	..	..	..	..	..	..	3-18‡
145—I	**	..	..	..	..	..	..	..	..	3-18‡
159—I	**	***	..	..	..	..	..	..	..	4-8‡
164—I	**	*f	***e	..	..	..	..	..	..	5-15‡
200—I	*	**f	***f	***f	..	..	..	..	..	9-6S†
210—I	**	**	**ee	**	***ef	..	..	..	..	7-14‡
232—I	*	**	**	**e	**f	..	..	..	..	9-6S†
235—I	l	..	..	..	..	..	..	..	..	3-11‡
271—I	**	**f	..	..	..	..	..	..	..	4-18†
277—I	*e	**e	**	**f	..	..	..	..	..	6-8‡
300—I	*	*f	*	*	..	..	..	..	..	7-11†
348—C	*	*e	*	*ef	*f	..	..	..	..	9-6S†
355—C	*	*e	..	..	..	..	..	..	..	4-13†
358—C	**	**	*	*e	of	**	**	**	**	12-13K†
370—C	*	*f	*	*f	..	..	..	..	..	6-5†
389—C	*	**	*	***e	**ef	**	**	..	..	10-16†
392—C	**	*	**	*f	*f	Chicken lost	..	..	..	2-1-45K†

o=No lymphoid accumulations present. *=Small lymphoid accumulations present. **=Moderate lymphoid accumulations present. ***=Large lymphoid accumulations present. l=Lymphomatosis. e=Eosinophils present in lymphoid areas. ee=Numerous eosinophils present in lymphoid areas. f=Liver very fatty. K=Killed on date given at termination of experiment. S=Male sold on date given; apparently normal when sold. I=Inoculated chicken. C=Control chicken. Op.=Operation. †=Absence of visceral lymphomatosis *post mortem*. ‡=Presence of visceral lymphomatosis *post mortem*.

to death than was found in the majority of birds which did not develop lymphomatosis. In a few of these same positive cases, samples of the liver taken at death still showed no marked lymphoid infiltration of the liver, the lymphomatotic lesions being confined to other organs. But in the majority, marked lymphomatotic infiltration of the liver had occurred within the month preceding death.

In several birds where the progressive increase in the amount of lymphoid tissue in the liver over a period of months approached, and in a few cases definitely reached, a lymphomatotic condition, a later regression in the amount of lymphoid tissue occurred, and a clinical lymphomatosis never appeared. In a few cases, notably 55, 185, and 206 (all inoculated birds), the peak of lymphoid accumulation was so definitely lymphomatotic that the regression was suggestive of recovery from mild cases of visceral lymphomatosis. In several others, notably 65, 229 (both inoculated birds), and 389 (a control), the degree of lymphoid infiltration never became what was considered definitely lymphomatotic but bordered upon it. In all such cases, a marked infiltration of eosinophilic leucocytes into the lymphoid areas, particularly during the regression period, was very noticeable. This was particularly true in the cases of recovery from a definite lymphomatosis. On the other hand, some eosinophilic infiltration into the perivascular lymphoid areas (but rarely so marked) was commonly observed throughout the series of liver samples studied, in both negative and positive cases and in varying degrees. The possibility that such eosinophilic infiltration might result from surgical trauma has been considered, but is

TABLE 4—Results of Liver Biopsies—Group 4

Chicken no. and status	Op. 1	2	3	4	5	6	7	8	9	Date of death or sale
	Date 3-17	4-14	5-12	6-13	7-21	9-15	10-13	11-15	12-13	
					Microscopic findings					
11—I	o	**	**	..	..	..	..	..	..	5-23†
14—I	**	*	***ee	**e	**f	**	Lost	..	..	1-25-45K‡
47—I	**	..	..	..	..	..	..	..	..	3-20‡
51—I	**e	*f	*ef	*ef	**f	*f	..	..	..	9-15†
68—I	*	**ef	**e	**	*	..	..	..	..	9-6S†
95—I	**	**	**	**	**f	..	..	..	..	8-16†
114—I	***e	..	..	..	..	..	..	..	..	3-27‡
127—I	*	..	..	..	..	..	..	..	..	4-1‡
141—I	**	**	***ef	**e	..	..	..	..	..	7-18‡
163—I	*e	*e	*	*e	*	..	..	..	..	7-21†
178—I	*	**	***	..	..	..	..	..	..	5-17‡
199—I	*e	**	**	..	..	..	..	..	..	6-3†
206—I	*	*f	*	**eef	*f	***f	lf	***	**	12-13K‡
223—I	**	**ef	***	**e	..	..	..	..	..	6-14‡
244—I	*	*f	**	**e	*f	**f	**ef	l	***	12-13K‡
257—I	*e	*	*f	*f	*f	*f	..	..	..	9-16†
258—I	***	**	**	l	..	..	..	..	..	6-21‡
281—I	***e	**	***ef	**e	***f	lf	l	l	***	12-13K‡
340—C	*	**f	**e	*	*	*f	*f	*	*	11-15†
349—C	**e	**	..	..	..	..	..	..	..	5-2†
374—C	*	of	*	*f	..	..	..	..	..	6-24†
387—C	*	*	**	**e	*	..	..	..	..	9-6S†
403—C	**	*	**ef	**ee	**eef	..	..	..	..	9-6S†
422—C	**	**f	*ef	*e	*f	*	*f	**	*	12-13K‡

o=No lymphoid accumulations present. *=Small lymphoid accumulations present. **=Moderate lymphoid accumulations present. ***=Large lymphoid accumulations present. l=Lymphomatosis. e=Eosinophils present in lymphoid areas. ee=Numerous eosinophils present in lymphoid areas. f=Liver very fatty. K=Killed on date given at termination of experiment. S=Male sold on date given; apparently normal when sold. I=Inoculated chicken. C=Control chicken. Op.=Operation. †=Absence of visceral lymphomatosis *post mortem*. ‡=Presence of visceral lymphomatosis *post mortem*.

believed unlikely because of the nature of the technique and type of instruments used in removal of the liver samples. Moreover, eosinophilic infiltrations are frequently noted in organs which have not been subjected to trauma.

Many of the liver samples studied showed marked fatty infiltration. In some birds, including both negative and positive cases, the amount of fat present fluctuated greatly from month to month. The impression was gained that this fluctuation might be related to the productive cycle, but the observations made were too incomplete to justify the drawing of any definite conclusions. No correlation between the amount of fatty infiltration and the degree of lymphoid accumulation was apparent.

DISCUSSION

The group of 96 chickens from which liver samples were collected monthly by biopsy over a period of ten months included not only birds inoculated with lymphomatotic material, but also control birds. In both of these groups of birds, some developed visceral lymphomatosis, while others remained negative. At least small, and in many cases moderate and even large, mostly perivascular, lymphoid accumulations in the liver were found in all but a very few of the liver samples studied—from control as well as inoculated birds, and negative as well as positive cases. This suggests that such lymphoid areas, particularly when they are perivascular and well-defined without diffusion outwardly, are normal for chickens and, within moderate limits, are not a manifestation of incipient lymphomatosis. These observations may throw some light on the question posed by Waters and Prickett[30], who point out that "the presence of microscopic lymphoid-like nodules diffusely spread throughout the tissues of apparently normal birds raises the important question as to whether such nodules represent normal, active lymphoid tissues or constitute a specific disease condition." On this same question, Gibbs and Johnson[3] have said: "Histological studies indicate that the fowl does not possess lymph glands and lymph nodes anatomically and physiologically comparable to those of higher animals, but does possess relatively large amounts of lymphoid tissue in the bone marrow, liver, spleen, and in certain locations along the digestive system. This tissue is closely associated with the vascular system by means of capillaries. In lympholeukosis [*i.e.*, visceral lymphomatosis] this lymph tissue appears to be in a state of hyperplasia, and the number of lymphoblasts in the tissue and capillaries is materially increased over that in the healthy fowl."

The appearance among the birds studied of several cases in which death from visceral lymphomatosis occurred within three or four weeks after the previous liver sample taken by biopsy had shown no marked lymphoid accumulations suggests that fatal cases may develop very rapidly. Some of these cases showed markedly lymphomatotic livers at death, but in others the lesions were confined to other organs, and the liver was still relatively normal at death. In a practical application of liver biopsy technique to diagnosis for the purpose of control of visceral lymphomatosis, such cases where the liver is never affected would be overlooked. Thus, the practical value of such biopsy technique as a routine diagnostic procedure would be somewhat limited. Its value probably lies principally in use for experimental work, and in diagnosis only in special cases.

The occurrence in some birds of a progressive increase to a marked, and in some cases even definitely lymphomatotic, lymphoid infiltration in the liver, followed by a gradual regression to an apparently normal condition, suggests that recovery may occur in some mild cases of visceral lymphomatosis which never become clinically apparent. The presence of unusually large amounts of eosinophilic infiltration in the lymphoblastic areas in such cases may or may not indicate that such eosinophilic leucocytes play some protective rôle or some active part in combating developing

lymphomatosis. It is interesting to note in this connection that Gibbs[2] found that, in neurolymphomatosis, examination of the blood showed that "differential counts indicate that the first increase is in eosinophile leukocytes, while the last increase is in small mononuclear lymphocytes. Sometimes the leukocytosis and the lymphocytosis are found overlapping." Oberling and Guerin[7] also report that in erythroblastosis the tumorous tissue is frequently infiltrated with eosinophilic leucocytes. In man, La Manna and Borghetti[5] reported that an eosinophilia varying from 6 to 24 per cent (average 8 to 10%) was observed in 36 of 560 patients with malignant tumors, and considerable tumor infiltration with eosinophils was found in 9 of 100 cervical cancers, while the number of eosinophils found histologically in the tumor mass of a fibro-angioneuroma associated with eosinophilia was striking.

SUMMARY

(1) A moderate amount of lymphoid infiltration in the liver of chickens is apparently normal, particularly when the areas are perivascular and well-defined without outward diffusion, and does not necessarily indicate a pathologic condition.

(2) In these studies, fatal cases of visceral lymphomatosis developed very rapidly; in some cases, which showed definite liver lesions at postmortem examination, death occurred in three or four weeks after biopsy had showed the liver to be apparently free of an abnormal amount of lymphoid infiltration.

(3) Recovery (or regression) occurred in mild cases of visceral lymphomatosis which had progressed to the point where they showed definite liver lesions microscopically in liver biopsy samples, but were not apparent clinically.

(4) Eosinophilic leucocytes appeared in many cases in both normal and lymphomatotic perivascular lymphoid nodules in the liver. They appeared in unusually large numbers in some cases which apparently showed recovery from mild visceral lymphomatosis, and may or may not play some protective rôle or active part in combating developing lymphomatosis.

(5) Liver biopsy technique is apparently of limited value as a standard diagnostic technique for the control of visceral lymphomatosis, since many cases were observed which showed no abnormal lymphoid infiltration of the liver microscopically at any time, the lesions being limited to other organs. These studies demonstrate that it has a definite use in experimental work, and possibly a limited use in diagnosis in special cases.

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Adsorbate Newcastle Disease (Avian Pest) Vaccine

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THE SEARCH for a biologic agent that can be used for prophylaxis against Newcastle disease [pneumoencephalitis] infection dates as far back as 1928, scarcely a year after the discovery of this disease in the Philippines. Farinas[1] prepared 17 types of Newcastle disease vaccine, the best one of which protected only 18 per cent of the birds injected. Topacio and Coronel[2] prepared 25 types of tissue and virus vaccines, the most potent of which immunized only 23 per cent of the test birds. The cultivation of the virus in developing chicken and duck embryos by Generoso[3] paved the way for the preparation of more potent vaccines. Acevedo and Mendoza were able to prepare a dye-treated, and later a glycerinated, vaccine which was used quite extensively in the field. The results of laboratory and field trials of these vaccines were, however, still variable. Some serials were fairly satisfactory while others were completely nonpotent. The present paper deals with the various experiments leading to the development of a new and better type of Newcastle disease vaccine.

The general principles of attenuating bacteria were followed in attenuating Newcastle virus in an effort to produce a satisfactory vaccine. Thus, attenuation of the virus by adaptation to particular tissues or hosts, by aging of the virus suspended in phosphate buffers of varying pH values, by keeping the suspensions in the refrigerator for varying periods, by desiccation, and by chemical treatment were tried.

ATTENUATION OF THE VIRUS BY ADAPTATION TO EMBRYONIC TISSUES

Serial passages of the virus in embryonic tissues were made with the object of adapting it to such tissues and finding out whether such adapted virus can be used safely as a vaccine. Samples of the virus from each serial transfer were tested for virulence on fullgrown, susceptible, White Leghorn chickens by injecting 1 cc. of each of the following dilutions of the virus in physiologic saline solution: 1 : 10 billion, 1 : 20 billion, and 1 : 40 billion. Chickens surviving the injection of the virus and nonvaccinated controls were later exposed to sick birds, previously given the virus, to test their immunity.

Results from these trials indicate that a few transfers fixed the virulence of the virus in embryos by invariably killing them within forty-eight to seventy-two hours, but 100 or more serial passages did not attenuate sufficiently to make it a safe vaccine for adult birds. Samples taken from the early, as well as from the later, passages possessed the same virulence for mature test birds. Birds surviving the injection of the virus proved to be immune to subsequent contact infection. This method of immunization was abandoned since, even with the highest dilution of 1 to 40 billion as the immunizing dose, a large number of the inoculated birds succumbed.

ATTENUATION BY AGING THE VIRUS

Definite amounts of the virus consisting of embryonic fluids of artificially infected embryos were suspended in phosphate buffers varying in pH values from 5.0 to 8.4. These suspensions were sealed and kept at temperatures ranging from 0 to 5 C., and samples from each suspension were tested for virulence at varying intervals. Results from one trial showed that the virus kept at pH 7.0 to 7.4 remained infective up to nine months of storage, while samples of the virus suspended at the range of pH 5.0 to 5.8 and those at pH 7.6 to 8.4 were

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no longer infective. Test birds inoculated with these innocuous samples proved to be susceptible to subsequent virus inoculation. This showed that this method cannot be used for attenuating the virus for vaccine purposes.

ATTENUATION OF THE VIRUS BY DESICCATION

Following the principle used in the attenuation of fowlpox and rinderpest viruses, attempts were made to attenuate Newcastle disease virus by desiccation. Artificially infected embryos were gathered, ground in a colloid mill, and the ground tissues dried over calcium chloride in a desiccator. Samples were taken at varying intervals, ground into fine powder, suspended in 20 per cent glycerin or 10 per cent gelatin solution, and their infectivity tested. Birds surviving the tests were later given fresh virus to test their immunity.

Results from three trials showed that the virus remained virulent for from three and a half to five months. After that period up to six months, the virus remained alive, causing slight reaction among the inoculated birds. After seven months, the virus was completely noninfective, but the immunity among the test birds was irregular. Powdered vaccine did not remain long in suspension but settled down in the bottle or in the barrel of the syringe, forming a rather sticky or gummy material. Because of these difficulties and the length of time required for attenuation, this method was given up in the meantime.

ATTENUATION OF THE VIRUS BY CHEMICAL TREATMENT

Using chloroform, ether, toluol, or formalin as attenuating agents, with glycerin-saline, fresh egg yolk emulsion or alumina cream in phosphate buffer, or distilled water as vehicles, emulsifiers, suspending fluids, or adsorbents, preliminary trials were made to determine which agent or combination of agents would be useful in attenuating the virus without destroying its antigenic properties. Many trials were made before experiments narrowed down to the following:

1) Equal parts of ground embryonic tissues and fluids from the same harvest were suspended in an equal quantity of buffered saline. Four aliquots were treated with 0.75 per cent chloroform, 5 per cent ether, 5 per cent toluol, and 0.1 per cent formalin, respectively. These treated aliquots were all placed in the refrigerator for twelve to fourteen days, shaking them at least three times a day. At the end of the period, they were tested for safety and potency, using 3 birds for each lot.

With the chloroform-treated vaccine, only 1 test bird survived; etherized vaccine was still infective and lethal; with toluolized vaccine, only 1 bird survived; while with the formalinized vaccine, 2 birds were immunized. The experiment was repeated using other lots of vaccine prepared in exactly the same manner. Practically the same results were obtained when tested, except that all 3 test birds for the toluol treated lot succumbed this time.

2) The composition of the next series of vaccines prepared was modified by introducing fresh, egg-yolk emulsion in phosphate buffer in the mixture to replace buffered glycerin as an emulsifier. The proportion of embryonic tissues and fluids to the various chemical attenuating agents, however, remained the same as in the first series. Using two sets of these series and increasing the number of test birds to 4 for each lot, the potency tests showed that replacement of buffered glycerin with fresh, egg-yolk emulsion did not improve the potency or the protective value of the different vaccines, although the formalin-treated lot again showed a decided advantage over the rest.

3) In the third series, the composition of the different vaccines was again modified. Alumina cream (alumina gel) emulsified in phosphate buffer or in distilled water, pH 7.2 to 7.4, was substituted for the fresh, egg-yolk emulsion as a protective or emulsifier and adsorbent. The other ingredients and their respective amounts remained the same as in the first and sec-

ond series. Potency tests fourteen days after preparation showed very marked improvement of the potency of each type of vaccine except the etherized lot which remained still infective. The chloroform-treated and the toluolized vaccines protected 50 per cent of the test birds which had been increased to 4 for each lot, while the alumina cream formalinized vaccine protected all the test birds. This experiment was repeated half a dozen times using new batches of the various vaccines, and the results proved to be consistent; that is, alumina cream, formalin-treated vaccine always gave the highest percentage of protection. Work was, therefore, concentrated on this type of vaccine, which the writer proposes to call an "adsorbate" Newcastle disease vaccine.

TABLE 1—Comparative Potency Test of Adsorbate and Glycerinated Vaccine

Serial no.	Alumina cream formalin-treated Nc vaccine (adsorbate)	Glycerinated vaccine
1	Potent	Partly potent
2	Potent	Potent
3	Potent	Partly potent
4	Not Potent	Not potent
5	Potent	Potent
6	Potent	Potent
7	Potent	Not potent
8	Potent	Potent
9	Potent	Potent
10	Potent	Potent
11	Potent	Potent
12	Potent	Not very potent
13	Potent	Not potent
14	Potent	Not very potent

Note—Test for each serial had 1 or 2 controls. All controls died of Newcastle disease infection in record time. Potent=Both test birds immunized. Not potent=Both test birds died of Newcastle disease. Partly potent=one test bird died of Newcastle disease. Not very potent=One test bird became sick but recovered.

ALUMINA CREAM, FORMALIN-TREATED VACCINE (ADSORBATE VACCINE)

Encouraged by the excellent results obtained in the foregoing preliminary experiments using alumina cream and formalin as the principal reagents in the preparation of the vaccine, more serials of this type were prepared, methods of preparation improved, and finally standardized and adapted throughout. The method of preparation is so similar to that used elsewhere that it is unnecessary to describe it again here.

Fourteen serials of the adsorbate vaccine and an equal number of the glycerinated vaccine were prepared from aliquots of tissues and fluids from the same harvest, and their protective powers compared, the dose of the vaccine, the test virus, and other conditions being the same. The results of the comparative test are shown in table 1.

The results of this comparative test clearly indicate that the adsorbate vaccine is just as good, if not better, than the glycerinated vaccine; for out of 14 serials of the formalinized vaccine only one was not protective, 13 were potent, solidly protecting all the test birds, while out of the 14 parallel serials of the glycerinated, three were not protective at all, two protected only half of the test birds, and two serials afforded only partial protection as one of the test birds in each case showed clinical symptoms after virus inoculation.

INFLUENCE OF ALUMINA CREAMS ON POTENCY OF THE VACCINE

The available brands of alumina cream (Eimer & Amend, and Laboratory) were tried so that a substitution could be made, should the supply of either run short. Three batches of vaccine for each brand were prepared and tested; the embryonic materials, the method of preparation, testing, and other conditions relative to the test were the same. The results of this comparative test showed both brands equally effective in that 400,000 m.l.d.'s immunized test birds whether 2 cc., 1 cc., or 0.5 cc. of vaccine was used. All control birds succumbed.

MINIMUM PROTECTIVE DOSE OF ADSORBATE VACCINE

In order to determine the minimum amount of vaccine necessary to protect

against 1,000 to 400,000 m.l.d.'s, varying amounts of a batch of vaccine of known potency were given to many test birds and then, two weeks later, each bird was given 1 cc. of the given dilution of freshly titrated virus. The test birds were all adult, susceptible, White Leghorn chickens. Results of the test are shown in table 2.

were injected. In addition, 3 other chickens were given 0.5 cc. each of a portion of the original untreated materials. Two weeks later, all the chickens together with 1 control were given 400,000 m.l.d.'s of the test virus. Results are shown in table 3.

It will be seen from the foregoing table that increasing the bulk or volume of a

TABLE 2—The Results of the Determination of the Minimum Protective Dose of Adsorbate Vaccine

No. chickens used	Dosage of vaccine per bird	Amt. test virus given (m.l.d.'s)	Results
5	0.2 cc.	400,000	1 developed clinical symptom but recovered. 1 had slight somnolence which lasted for three days. 3 had no reaction (immune).
5	0.25 cc.	400,000	1 had slight somnolence which lasted for three days. 4 had no reaction (immune).
5	0.5 cc.	400,000	1 had slight somnolence which lasted only for one day. 4 had no reaction (immune).
69	1.0 cc.	1,000-400,000	All with no reaction (immune).
22	2.0 cc.	1,000-400,000	All with no reaction (immune).

Controls given 2 m.l.d.'s (1 cc. of 1:20,000,000,000) all died of Newcastle disease in record time.

Table 2 shows that the minimum protective dose of the adsorbate vaccine is 0.25 cc. As no experiment could be conducted to determine the minimum dose for younger birds (for lack of experimental chickens), it was recommended that the amount of 0.25 cc. be used for chickens from 4 months to 6 months old, 0.5 to 1 cc. for pullets and cockerels, and 1 to 2 cc. for adults. Field trials up to the present indicate that these are satisfactory and may, therefore, be the proper dosages.

Effect of Increased Amount of Diluent on the Potency and Resulting Immunity

In order to determine the influence of bulk of a given dose of the vaccine upon the immunity induced, 0.5-cc. amounts of a serial of vaccine of known potency were treated as follows: One portion was centrifuged at high speed to settle the tissues from the diluent and the latter poured off. One-tenth and 0.25 cc. was diluted with the minimum amount of saline to allow the injection. The other samples were diluted with saline to increase the volumes to 2.5, 5.0, and 10 cc., respectively. Then for each aliquot, 3 susceptible chickens

given dose without increasing the actual content of antigenic material did not influence the potency of the vaccine or the resulting immunity induced by it. It was evident that the antigenic part of the vaccine was solely responsible for the production of immunity; and also that the amount of protection it could induce had certain limit and that limit could not be enhanced by increasing the bulk of the inoculum through the addition of an inert diluent.

Length of Immunity Produced by the Adsorbate Vaccine

It is necessary to determine the length of immunity that the vaccine will produce in order to find how many times a year a given flock must be vaccinated. Freshly prepared lots of vaccine were pooled and tested for potency. When found potent a number of birds were injected and divided into four groups of 3 birds each. These groups were inoculated with a test dose of the virus at varying periods ranging from two to three and a half months after the vaccine injections. (Due to lack of test birds three and a half months was the longest period tested; work along this

line, however, will be continued.) Results of the above test showed that all vaccinated birds withstood a test injection of 200,000 m.l.d.'s while all controls succumbed to 10 m.l.d.'s. Pending further work on this phase, it seems safe to recommend that flocks should be vaccinated three or four times a year.

TABLE 3—The Effect of Volume of a Given Dose Upon the Potency of the Vaccine and the Resulting Immunity

Volume into which aliquots 0.5 cc. of vaccine were converted	No. chickens used	Results
0.1 cc. centrifuged sediment diluted to 5 cc.	3	1 marked symptoms 1 slight symptoms 1 immune
0.25 cc. centrifuged sediment of 0.5 cc. of vaccine	3	All immune
0.5 cc. diluted to 2.5 cc.	3	All immune
0.5 cc. diluted to 5 cc.	3	All immune
0.5 cc. diluted to 10 cc.	3	All immune
0.5 cc. untreated vaccine	3	All immune
Control		Died of—Newcastle disease

KEEPING QUALITY OF THE VACCINE

Low Temperature.—In order to determine how long the vaccine can be kept at low temperature without losing its potency, a lot of potent vaccine prepared from tissues and fluids of the same harvest was stored in a refrigerator whose temperature ranged from 0 to 5 C. At intervals of one month, two months, and two and a half months, and depending upon the number of available susceptible chickens on hand at the time, its potency was tested by injecting the birds with 1 cc. of the vaccine intramuscularly. Fourteen to twenty-one days after the vaccine injection, the test birds together with 1 control were given 1 cc. each of a dilution containing 1,000 m.l.d.'s of a freshly titrated virus. Another lot of vaccine from a different batch of tissues and fluids was similarly prepared, stored, and tested, although the third test had not been completed because the war had already broken out before the vaccine birds received the virus inoculation. The results of potency tests of the two serials so far tested show that the potency of the adsorbate vaccine was not materially altered after two and a half months of storage at 0 to 5 C., the longest period so far tested. However, 1 bird in the two-month potency test and 1 bird in the two and a half-month potency test showed somnolence for three and two days, respectively, but both recovered completely. Work on this phase will be continued.

Room Temperature.—The question of how long the vaccine can be kept at ordinary room temperature was also studied. Hence, a batch of vaccine prepared from the same batch of tissues and fluids was stored in a dark cabinet kept in a room where the temperature ranged from 27 to 28 C. At intervals of four, eight, fourteen, and twenty-one days after storage, its potency was tested on available test birds. Two more separate experiments of the same nature were conducted, using different serials of potency vaccines as well as separate sets of experimental chickens. The composite results of these three experiments show that all the three serials remained potent after four days of storage at 27 to 28 C., while after the eighth day, 3 test birds came down and died of the disease after the virus inoculation while 6 birds were immune. After the second week of storage, results showed that 4 test birds succumbed and 6 survived. After the third week of storage, the results were again the same; 6 birds were protected while 4 succumbed. Work on this phase will also be continued.

EFFECTS OF pH UPON THE POTENCY AND KEEPING QUALITY OF THE VACCINE

Although no controlled experiments have actually been conducted, it was possible to determine the pH of the different serials of experimental vaccines and to show that potent serials varied from pH 6.4 to pH

7.2. It seems, therefore, that pH has little effect on the potency of this vaccine. Similarly, no controlled experiments had been conducted to determine the effect of pH on the keeping quality, but the five serials which had been used in the determination of the keeping quality at refrigerator and room temperatures seemed to show that pH not very far from neutrality (pH 6.8 to 7.2) seemed to favor longer keeping quality of the vaccine. No determination of effect of storage on the pH values was conducted, but this will be done.

DOUBLE EXPOSURE TEST FOR POTENCY OF THE VACCINE

Before field trials of the vaccine were allowed, a severe test for its potency was performed. One hundred chickens were divided into two groups of 50 birds each. One group was given a dose of 1 cc. and the other 2 cc. of a potent vaccine. After fifteen days, half (25) of the first group and half (25) of the second, together with a control, were given 40 m.l.d.'s of a freshly titrated virus, while the remainder of both groups, together with another control, were given 1,000 m.l.d.'s of the same virus. Two normal controls were added to the flock. When the 2 inoculated controls showed symptoms of the disease they were not removed from the flock, thus exposing the vaccine-test birds to double risks, one from the inoculated virus, the other from contact infection with the sick birds.

The results were amazingly satisfactory. Three birds developed slight clinical symptoms but soon recovered; all the rest (97) were solidly protected. The conditions under which this test was conducted were more severe than ordinarily found in the field, *i. e.*, all the vaccine birds definitely got the virus, no removal of sick fowl or segregation had been made, and no disinfection had been instituted; the enclosure holding the birds was small so that exposure to infection was severe. In spite of this, all the test birds proved to be immune.

FIELD APPLICATION OF THE VACCINE

Eighteen serials consisting of 75,000 doses of this vaccine have been used by veterinarians for controlling Newcastle disease infection in poultry projects around Manila and in the provinces of Rizal, Bulacan, Pampanga, Tarlac, and Pangasinan. Both exposed and nonexposed flocks were vaccinated and the results have been satisfactory. There was not a single instance in which the vaccine itself caused the infection. Losses in newly infected flocks were minimized after the administration of the vaccine. In one infected flock, however, cases continued to register even two weeks after the vaccination, and losses were heavy. This flock had a mixed infection to start with, that of Newcastle disease and roup. Where the vaccine was used before exposure, the results were very satisfactory. Where the birds ran at large after vaccination, as in some rural districts, evaluation of the results could not be properly made. According to field veterinarians, however, results were satisfactory, for some owners testified that their chickens remained healthy in spite of the presence of infection in their vicinity. Nevertheless, not until properly controlled field tests can be conducted will its efficacy be definitely established.

DISCUSSION

Four methods of attenuating the Newcastle disease virus were tried, namely, adaptation to the embryonic tissue, aging the virus suspended in phosphate buffer of varying pH values and keeping the suspensions at low temperature for varying periods, desiccation, and chemical treatment. The first method proved to be a failure because the virus remained infective even at very high dilutions. Although it was possible to immunize a certain number of birds with a subinfective dose of a very dilute virus, the method proved too dangerous, considering the difficulty of standardizing the virus content of a tissue suspension and the fact that different individuals vary in susceptibility.

The second method also proved to be a failure because pronounced acid and alkaline solutions seemed to destroy the virus and its antigenic properties. Neutral and slightly alkaline solutions, on the other hand, seemed to favor long survival of the virus, but failed to attenuate it. Birds given noninfective virus suspensions proved to be still susceptible to subsequent virus inoculation, and it may be inferred that the virus particles in the suspension were completely destroyed, if immunity is to be linked with the sojourn in the body of an attenuated or living virus.

Although attenuation by desiccation may be utilized in the preparation of this vaccine, the long period necessary to accomplish the purpose, plus the fact that the virus is difficult to attenuate to a point where it will not cause visible symptoms without sacrificing uniformity of its protective value, make this method also unsatisfactory. Furthermore, the difficulty of making a uniform suspension of the powdered tissues and the sticky character of the material render injections difficult and dosages inaccurate.

The chemical method of attenuation, using alumina cream as an adsorbent and emulsifier, with formalin as an inactivating agent, has given surprisingly remarkable results. Whether any of the virus particles had been adsorbed into the alumina cream cannot be ascertained as no elution attempt had been made. It was certain, however, that not all the virus particles were adsorbed into the alumina cream in the proportion in which it was used, for injection of a nonformalinized mixture caused slight manifestation of the disease. The mechanism with which the vaccine worked effectively may be summarized as follows.

The unadsorbed virus particles were attenuated (not killed) by the formalin to the point where they no longer caused infection, whereas those adsorbed in the colloid particles of the alumina cream were not affected by it. When the vaccine was injected, the virus particles were released gradually from the colloid particles in subinfective doses, inducing the necessary antibody response (immunity) without causing any visible clinical manifestations.

The main properties of the adsorbate vaccine, namely, its potency and keeping qualities, are by no means perfect and need further improvement.

In the course of the various experiments — particularly in the adaptation of the virus to embryonic tissue, in the aging of the virus suspensions in the refrigerator, and in the testing of desiccated virus—test birds which reacted but recovered proved to be immune to subsequent virus inoculation. With that observation as a basis, all available test birds on hand at that time were properly identified, and those which had received the virus from a month and six days to a year previously, were subjected to an immunity test and all proved to be immune. Work on this method of immunization was therefore started in order to accumulate more data but was cut short by war. A separate report will be made as soon as sufficient data become available.

Likewise, it had been observed that a number of susceptible test birds given a known quantity of diluted or desiccated virus showed no reaction at all and yet proved to be immune to subsequent virus exposure. Similarly, all the adsorbate-vaccine birds showed no visible reaction to vaccine injection and proved to be immune when given the test virus. It may be assumed that the diluted virus contained only subinfective doses. In the case of the dry and the adsorbate vaccines, it may be asked whether the virus was actually dead or alive. As there is no available technique which can definitely settle this point, it may be inferred that the virus is attenuated but not dead. (Virus heated at a high temperature or treated with high concentrations of chemicals is killed (dead) and rendered nonantigenic.)

SUMMARY AND CONCLUSIONS

In an effort to develop an effective vaccine against Newcastle disease [pneumoen-

cephalitis] four methods were tried, namely, (1) adaptation to embryonic tissues of the host; (2) aging the virus suspended in phosphate buffer of varying pH values; (3) attenuation by desiccation; and (4) attenuation by chemical treatment. Of the four, only the chemical method developed a highly potent adsorbate vaccine, 75,000 doses of which have been dispensed and used for controlling Newcastle disease infection in the field.

From the various experiments conducted, the following conclusions were drawn:

1) Newcastle disease virus was readily adapted to embryonic tissues of its host, but its virulence was not attenuated or modified to make it a safe vaccine for adults. However, birds surviving injections of diluted virus, especially those which showed a reaction, were immune to contact infection.

2) Aging the virus suspended in phosphate buffers of varying pH values failed to attenuate the virus. Storage to a point where the virus was rendered innocuous caused the material to lose its antigenicity.

3) Attenuation by desiccation, besides being a long process, produced a vaccine of uncertain safety, variable antigenicity, and unsatisfactory physical properties.

4) Chemical treatment, using alumina cream as an adsorbent and emulsifier and formalin as an attenuating agent, proved to be the most effective method of producing a highly potent Newcastle disease vaccine with the following characteristics:

a) It is more potent than glycerinated vaccine.

b) Its minimum protective dose is 0.25 cc.

c) The immunity induced by it endures at least three and a half months, the longest period so far tested.

d) Its keeping quality at low temperature (0 to 5 C.) is two and a half months, the longest period so far tested.

e) It could be stored safely at room temperature for four days without loss of potency; after eight days, it lost 50 per cent of its protective power, while one extraordinarily potent serial remained protective even after twenty-one days.

f) It seemed that pH had little effect on potency, while pH not far from the point of neutrality (pH 6.8 to 7.2) seemed to favor longer keeping quality of the vaccine.

5) Increasing the bulk of the vaccine through the addition of an inert diluent did not influence the immunity produced by the vaccine.

6) Injecting one, two, or more doses of adsorbate vaccine followed by 40 to 1,000 m.l.d.'s of the virus conferred an almost solid protection to natural infection.

7) Birds previously inoculated with virus, and which did not come down with it, were immune to subsequent virus inoculation. A separate report on the method of immunization will be made.

8) Field application of vaccine was highly satisfactory.

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Immunity and Cross Immunity Reactions Obtained with Several Avian Lymphoid Tumor Strains

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Review of the Literature

It has been shown that an avian lymphoid tumor[3] similar to other transplantable tumors was capable of imparting immunity in its host to a second implantation of homologous tumor tissue. Burmester and Prickett[2] found that the immunity appeared in all birds that survived an active growth, that it could not be overwhelmed by a very large dose or repeated doses of the same agent, and that it remained for a prolonged experimental period. Olson[10] suggested that the ability of the tumor to immunize against itself was enhanced by serial passage.

A similar immunity has been obtained with four other transplantable lymphoid tumor strains which were developed[1] directly from cases of naturally occurring lymphomatosis. Immunologic relationships among these several strains and with another lymphoid tumor strain[3] have also been investigated. Results of these studies are summarized in this report.

Materials and Methods

Birds used in this study were pedigreed White Leghorn chickens of the laboratory stock and were from genetic strains designated as relatively susceptible to lymphomatosis.

Five lymphoid tumor strains were used. Four of them, designated as RPL 14, 15, 16, and 17, and described by Burmester and Prickett[1], arose from tumorous organs of cases of naturally occurring, visceral lymphomatosis. Strains RPL 14, 15, and 16 were initiated by intraperitoneal inoculation of a saline suspension prepared from the lymphomatous tumors. After the second and third passages, the intramuscular route was used for their propagation. Strain RPL 17 was originated and maintained in serial passage by intraperitoneal inoculation of the cells obtained from tumorous livers. The fifth tumor strain, designated here as RPL 12, is similar to the above mentioned strains but was developed by Olson[8, 9] from a case having the characteristics of a lymphocytoma.

For each RPL strain, three different types of inoculums, described herewith, were used.

Cell Suspension.—A suspension of tumor cells, not previously frozen, was used for all birds in the serial passage of each strain. It was also used for those serving as controls for potency of the inoculum and for all birds that were reinoculated to test for the presence of an immunity. Such inoculums were prepared from muscular tumors or, in the case of RPL 17, hepatic tumors of birds in the serial passage of the respective strains. The tumor material was passed through a mincer[7], suspended in approximately three times its weight of 0.85 per cent NaCl solution, and filtered through a layer of sterile cheese cloth.

Cell Suspension, Frozen and Thawed.—The viability of strains RPL 14, 15, and 16 was tested after various intervals of storage at a low temperature. The tumor material, obtained from the same source as above, was frozen and stored by the procedure previously described[1]. While in the pyrex tube, the material was thawed in tap water (18 C.) and further processed by the procedure used in preparing the cell suspension above.

Cell-free Extract.—For the preparation of the cell-free inoculums, muscular and hepatic tumors obtained from birds in serial passage were ground to a smooth paste in a mortar with sterile sand. The material was suspended in three times its weight of saline and the cellular débris eliminated by somewhat different procedures. For strains RPL 15 and 17, the suspension was centrifuged for fifteen minutes in an angle centrifuge at 3,000 r.p.m. and the top half of the supernatant carefully syphoned off and used as the cell-free inoculum. For RPL 14, the suspension was passed thru a filter of sand superimposed with closely packed spun glass wool. For RPL 16, it was passed through the same type of filter and then centrifuged for five minutes at 3,000 r.p.m. and the upper half of the supernatant used. Although the examination of stained smears showed that none of the extracts contained whole cells, the above procedures did not eliminate their presence in the inoculums beyond a question of doubt. However, the probability of many viable cells being present in inoculums prepared by the above procedures is not great.

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All serial passage inoculations were made when the birds were from 1 to 26 days of age, with 0.1 to 0.5 cc. of inoculum injected intramuscularly for strains RPL 14, 15, and 16, and intraperitoneally for strain 17. Reinoculations were made after an interval of twenty-one to eighty-nine days with 0.5 cc. injected intramuscularly.

RESULTS

Results from reinoculations with the same tumor strain are summarized in table 1. Within each tumor strain, the results are segregated with respect to the type of material used in the first inoculation.

All birds surviving an inoculation with the cell suspension used in the serial passage of strains RPL 14, 15, and 16 were completely resistant to a second implant of the same strain. The induced immunity was not so uniform in the survivors of RPL 17, since 4 of 25 birds that were reinoculated developed tumors.

Birds that survived the inoculation with frozen cells were also resistant to implants of fresh suspensions. This was true despite the fact that with strains RPL 14 and 15, only 1/3 to 1/2 of the birds reinoculated had developed palpable tumors after the first inoculation.

The implantation of tumor cells in survivors of cell-free inoculations produced variable results. Four birds out of 10 survivors of cell-free RPL 14 inoculation grew local tumors when implanted with RPL 14 cells, whereas only 1 of 26 survivors of RPL 15 cell-free inoculation developed tumors when subsequently implanted with RPL 15 tumor cells.

All cell suspensions used for the second inoculations were tested for potency in birds not previously inoculated but of about the same age as birds in the experimental groups. As shown in table 1, all suspen-

TABLE 1—Results from Homologous Immunity Test

Type inoculum in 1st injection	1st inoculation		Interval between 1st & 2nd inoculation (days)	2nd inoculation with unfrozen cellular inoculum of same strain	
	No. chickens inoculated	No. with tumors		No. chickens inoculated	% with tumors
Tumor strain RPL 14					
Cell susp., not frozen	70	69	26-63	31	0
None (Control*)				7	100
Cell susp., frozen & thawed	30	11	24-32	29	10
None (Control*)				5	100
Cell-free extract	20	8	21	10	40
None (Control*)				5	100
Tumor strain RPL 15					
Cell susp., not frozen	89	85	30-89	29	0
None (Control*)				7	100
Cell susp., frozen & thawed	46	20	35-60	38	0
None (Control*)				11	100
Cell-free extract	31	3	61	26	4
None (Control*)				5	100
Tumor strain RPL 16					
Cell susp., not frozen	30	30	29-46	5	0
None (Control*)				7	86
Cell susp., frozen & thawed	10	9	60	9	0
None (Control*)				4	100
Cell-free extract	32	10	80	20	100
None (Control*)				4	100
Tumor strain RPL 17					
Cell susp., not frozen	103	72	46-95	25	16
None (Control*)				7	86
Cell-free extract	19	8	67	8	12
None (Control*)				7	86

*Control birds were inoculated for the first time with the cell suspensions used in testing the extent of immunity in birds previously inoculated.

sions were highly active as indicated by the high percentage of takes.

Birds that survived an implantation with one strain, or were proved immune to one strain by reimplantation tests, were injected with tumor cells of other strains. Results of these cross immunity tests are presented in table 2.

All of 19 birds surviving RPL 14 were completely resistant to implants of RPL 15, and all except 1 of 35 birds immune to RPL 14 were also immune to RPL 16; however, 2/3 of the RPL 14 immune birds, when inoculated with RPL 17, developed tumors. Of the 27, RPL 15 immune birds inoculated with RPL 16, only 1 developed a tumor. However, when similar RPL 15 immune birds were implanted with RPL 12 or RPL 17 tumor cells, many (76 and 90%, respectively) developed tumors. Survivors of RPL 16 were found to be immune to implants of RPL 15 but susceptible to RPL 17, and 82 per cent of the RPL 16 immune birds, when implanted with RPL 12, developed tumors. All 12 birds surviving RPL 17 were found immune to implants of RPL 15; however, when 48, RPL 17 immune birds were inoculated with RPL 12, 77 per cent developed tumors. Test showed that birds proved immune to RPL 12 were also completely resistant to implants of RPL 16. All inoculums for experiments reported in table 1 were tested for potency in the manner outlined.

It has long been known that normal tissues, especially spleen and embryo skin, when injected into susceptible animals, will increase their resistance to many but not all transplantable tumors[3]. As a check on the specificity of the immunity obtained with the lymphoid tumors reported herein, two experiments were conducted to test the ability of normal tissues to induce a resistance or immunity against the lymphoid transplants, similar to that obtained with the tumor itself.

In the first experiment, normal pectoral muscle and normal spleen were minced and suspended in 0.85 per cent NaCl solution. Each suspension was injected into the pectoral muscle of 9 healthy birds. A suspension of strain RPL 12 tumor cells injected into the opposite pectoral muscle fifteen days later produced large tumors in all birds. In a second test, a composite of normal thymus, bursa of Fabricius, spleen, and liver was minced and suspended in normal saline. One cc. of this suspension was injected into the pectoral muscle of 20 healthy birds 58 days of age. After eight days, the same amount of similar but freshly prepared normal tissue suspension was injected into the opposite pectoral muscle. Seven days later, a suspension of RPL 12 tumor cells was injected into 10 birds and a suspension of RPL 16 cells was injected into the remaining 10 birds. Again all birds developed large tumors, showing that no resistance was induced by the normal tissues against either of the tumor strains used. Thus, it may be concluded that the immunity obtained against the transplantable lymphoid tumor is induced by the tumor cell or its elements and not by the injection of normal tissue elements which may have been included in the tumor suspensions.

DISCUSSION

Results obtained in reimplantation tests with strains RPL 14, 15, 16, and 17 showed that birds surviving an implant of tumor cells are almost invariably immune to a second inoculation with homologous tumor tissue. The only exceptions occurred with RPL 17 in which 4 birds, of the 25 inoculated, developed tumors. Since all birds in the serial passage of RPL 17 were inoculated by the intraperitoneal route, the development of tumors could not be determined by palpation. Thus, the development and regression of tumors among the survivors are open to question, even though a high percentage of the chicks inoculated became positive (table 1). In contrast, all birds used in the serial passage of strains RPL 14, 15, and 16 were inoculated by the intramuscular route and tumors were palpable in all except 1 bird in each of strains 14 and 15. The solid im-

munity obtained is similar to that described previously[3] for RPL 12.

The frozen inoculums used in strains RPL 14 and 15 produced tumors in less than half of the birds inoculated, yet most of the survivors (100% for RPL 15) were resistant to a highly potent inoculum. Further evidence for the production of an immunity without gross tumor formation is found in the data of birds inoculated with cell-free extract of tumor strains RPL 14 and 15 (table 1). Not one of the birds that survived a cell-free inoculation developed a palpable tumor and only a few (5 of 36) showed tumor formation upon reinoculation with highly active cell suspension. A confirmation of this result was not obtained, however, in an experiment in which different lots of birds were given one to three injections of 0.5 cc. to 3 cc. of a tumor extract prepared with the aid of a Waring blender and centrifuge. When resistance was tested by inoculation with cell suspension of the same strain, birds that had received the tumor extract proved as susceptible as the birds in two sets of controls, one of which had received injections of extracts of normal muscle, and the second one had had no previous treatment. A similar result was obtained with RPL 16 in that none of the birds injected with the cell-free extract was resistant to a subsequent inoculation with a cell suspension.

Though somewhat inconsistent, strains RPL 14, 15, and 17 have given results indicating that under certain circumstances inoculums which produce no, or a low percentage of, local tumors will nevertheless induce sufficient resistance to prevent the growth of new tumors upon inoculation with cell suspensions, even though no palpable tumors were previously present in the birds.

Olson[10, 11] obtained a similar result with some of the early passages of his lymphoid tumor strain and with tumor materials that had been attenuated by freezing. However, in other experiments, he[11] found that when inoculums were centrifuged at various speeds and intervals the ability to induce immunity disappeared concomitantly with the loss of its capacity to produce primary tumors. The latter is in agreement with the findings of Burmester and Prickett,[3] which indicate that active growth of this tumor was a necessary adjunct to immunity against a second tumor growth.

The difference in results obtained between Olson[9, 10, 11] and Burmester and Prickett[3], and between the several strains, and within one strain (RPL 15) as reported herein, may be due to the inadequacy of the methods used in determining the presence of tumor growth or to differences in malignancy between strains or even within strains. The detection of tumors by digital palpation or by gross visible means is an arbitrary method depending largely on the size and location of the tumor. Small tumors, or reactions located deep in the pectoral muscle which regress and do not continue to grow, could easily escape detection, yet they might be of a character to provoke a refractory state which would prevent the growth of reimplants of certain tumor strains. Thus, inoculums containing only a few viable cells may have been responsible for tumors too small to be recognized by palpation,

TABLE 2—Results from Cross Immunity Tests

Survivors of strain-RPL	Immune to strain-RPL	Inoc. with strain-RPL	No. inoc.	% pos.
12	12	16	27	0
Control*		16	5	100
14		15	19	0
Control*		15	7	100
	14	16	35	3
Control*		16	6	100
	14	17	15	67
	15	16	27	3
Control*		16	6	100
	15	17	31	90
	15	12	58	76
Control*		12	4	100
16		15	13	0
Control*		15	7	100
16		17	4	100
	16	12	66	82
17		15	12	0
	17	12	48	77
Control*		12	8	100

*Control birds were inoculated for the first time with the cell suspensions used in testing the extent of immunity in birds previously inoculated.

but which rendered the birds immune to further implants.

Olson[9] has indicated that the malignancy of this tumor strain (referred to here as RPL 12) has increased with serial passage. This characteristic appears to have been further increased during its serial passage at this laboratory, since it was much more active when used by Burmester and Prickett[2] than in the early passages by Olson[9]. Burmester and Prickett[3] have indicated that RPL 16 appeared to be somewhat more malignant than RPL 14 and 15. There is some indication that refractivity provoked by an inoculum which does not necessarily induce gross tumor growth may be sufficient to protect against implants of homologous tumors of low malignancy, but active tumor growth would appear to be necessary to induce sufficient immunity against tumor implants of a high order of malignancy.

Results presented in table 2 indicate a rather close immunologic relationship between strains RPL 14, 15, and 16, whereas such a relation is not so apparent with RLP 12 and 17 either between themselves or with RPL 14, 15, or 16. The close relationship between RPL 15 and 16 was to be expected since they arose from the same (E1121A) original donor; however, the inoculums were prepared separately from tumorous spleen and liver, and were maintained separately thereafter[3]. The data show that most of the RPL 14, 15, or 16 immune birds were still quite susceptible to implants of RPL 17; and that RPL 15, 16, 17 immune birds were still susceptible to implants of RPL 12. However, RPL 17 immune birds were resistant to RPL 15, and RPL 12 immune birds were resistant to implants of RPL 16. Thus, in order to test the immunologic relationship between certain of the lymphoid tumor strains the tests must be made in certain sequences.

It appears that some lymphoid tumor strains are so nearly alike as to give a refractory reaction when the tests are made in either sequence. However, other strains differ sufficiently to give a refractory reaction only when tests are conducted in one sequence, and little or no resistance is demonstrated when the sequence is reversed. Lumsden[8] and others obtained similar results when working with mammalian tumors.

Oberling and Guerin[6], working with leucemia of chickens, obtained much greater resistance when the first inoculation was with a strain of high malignancy than with a strain of relatively low malignancy. A similar explanation may account for the results reported herein, since unpublished data of this laboratory indicate that RPL 12 at present is more malignant than strains RPL 14, 15, 16, or 17, though the relative malignancy of strain 17 is still questionable. It is believed that birds surviving an implant of a lymphoid tumor strain are highly resistant to other lymphoid tumor strains of equal or lower malignancy but are still relatively susceptible to implants of strains of higher order of malignancy.

Burmester, Prickett, and Belding[4] have demonstrated the presence of a filterable agent in the lymphoid tumor strain RPL 12 which causes osteopetrosis, and lymphomatous tumors in the viscera. Recent results with other RPL strains indicate the presence of a similar agent producing lymphomatous tumors in the viscera. It was also found[14] that birds immune to implants of RPL 12 tumor cells remained susceptible to the action of the agent. In working with sarcoma of the fowl, Rous[11] found two distinct sorts of resistance, one directed against the implanted tumor cells as such and the other against the action of the agent causing the neoplastic change. The results reported here presumably are due to an immunity directed against the implanted tumor cells, although variation in the action, or resistance to action, of the filterable agent may account for some of the apparent inconsistencies.

SUMMARY

1) Survivors of implants of the lymphoid tumor strains RPL 14, 15, 16, and 17, which were recently obtained from cases of naturally occurring visceral lym-

phomatosis, were immune to subsequent implants of homologous tumor tissue. The longest period tested was eighty-nine days.

2) Many chickens inoculated with frozen tumor cells or cell-free tumor extracts of strains RPL 14 and 15 did not develop palpable tumors and a high percentage of these survivors was found to be immune to implants of highly active cell suspensions.

3) Cross immunity was demonstrated between most of the avian lymphoid tumor strains investigated. In certain combinations, cross immunity was obtained only after the sequence of inoculation was reversed.

4) Injection of normal tissue suspensions did not increase measurably the resistance to implants of lymphoid tumor cells.

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Isolation of Shigella Gallinarum From Turkey Eggs

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Review of the Literature

The transmission of the fowl typhoid organism through the egg has been studied extensively. The work of Beaudette[1], Beach and Davis[2], Martinaglia[3], Komarov[4], Hinshaw and Taylor[5], and Johnson and Pollard[6] has incriminated the egg as a mode of transmission because the organism was found in the ovary and in chicks and poults less than 2 weeks of age. To our knowledge, the organism has not been isolated directly from the egg. The purpose of this work was to determine the frequency with which this organism, or members of the Salmonella group, could be isolated from incubated eggs from turkeys reacting to the tube agglutination test for pullorum disease. Considerable losses are experienced in poults as a result of fowl typhoid infection, so that such information should be valuable in formulating control plans.

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I am indebted to Dr. W. R. Hinshaw, University of California, Davis, Calif., who confirmed the identification of the organisms isolated from the egg, and to Dr. J. P. Delaplane, who, until his resignation Dec. 1, 1945, was poultry pathologist, Division of Veterinary Science, College Station, Texas, for his coöperation and generous assistance.

Materials and Methods

In January, 1945, 17 turkey hens, which were reactors to the tube agglutination test for pullorum disease and were members of two

flocks which had experienced fowl typhoid, were obtained to furnish fertile eggs for the study. The hens were mated to reactor toms and the eggs from these matings were placed in the incubator at biweekly intervals.

The eggs were allowed to incubate for fifteen days at 99.5 F., after which they were removed and placed in a refrigerator at 40 F. for forty-eight hours in order to kill the embryos. The eggs were returned to the incubator for a period of forty-eight hours to permit growth of any organisms before culturing.

The eggs were wiped with 95 per cent alcohol and the large end carefully opened. One loopful of the contents was aseptically removed and streaked on the type of nutrient agar used routinely in the isolation of *Salmonella pullorum* or *Shigella gallinarum*. Any suspicious organisms which were present in cultures were checked for identity by examining stained preparations and through the use of differential sugar mediums.

At the close of the experiment, the hens used in this study were killed and cultured for *S. pullorum* and *S. gallinarum*.

DISCUSSION

Three hundred and seventy-four eggs were collected, incubated, refrigerated, reincubated, and cultured on nutrient agar slants.

Several eggs with cracked shells were found to harbor Staphylococcus, Streptococcus, and coli organisms. These same organisms were present as occasional contaminants in eggs which did not show a visible crack. One infertile egg yielded a culture of an organism that was rod-shaped, bipolar, gram-negative, and produced acid, but no gas, in dextrose and maltose. Sucrose and lactose showed no change. This indicated that the organism was *S. gallinarum* or closely related to it. Thirteen other sugars were inoculated. Acid, but no gas, was produced in levulose, arabinose, and xylose. No changes were observed in salicin, inulin, esculin, and raffinose. This organism reduced nitrates to nitrites, did not form indol in tryptone broth, produced hydrogen sulfide in forty-eight hours on lead acetate agar, did not acidify or coagulate purple milk, and did not liquefy gelatin. These reactions are typical for *S. gallinarum*, and the organism was identified as such on this basis.

S. pullorum was recovered from 2 of the hens that were used for autopsy and cultured, but *S. gallinarum* was not recovered from any of the birds.

Since the completion of the above work, 12 additional reactors to the tube agglutination test for pullorum disease were submitted to this laboratory for bacteriologic examination and culture. These birds also had a history of exposure to typhoid infection and they originated near the birds used in the above experiment. If no *S. pullorum* was isolated from these birds, this flock would be considered as having had no reactors and could be nationally advertised as a U. S. pullorum clean flock under the National Poultry Improvement Plan.

These 12 birds (8 hens and 4 toms) were studied pathologically and bacteriologically. The liver, spleen, lungs, oviduct, ova, testes, caseous material in the serous cavities, and obvious lesions in other tissues were cultured on nutrient agar slants, to which brilliant green had been added at the rate of 1 : 100,000. In addition, the liver, heart, and spleen were ground together and 1 to 2 Gm. placed in tetrathionate broth, the remainder in beef infusion broth. The ovary was ground by using a mortar and pestle, then one loopful was streaked on brilliant green agar, one loopful put in tetrathionate broth, and the remainder placed in beef infusion broth. The oviduct was placed in tetrathionate broth.

After incubation for forty-eight hours, the slants were examined for growth. One cubic centimeter of the infusion broth, containing the tissues, was placed in tetrathionate broth and a small loopful on brilliant green nutrient agar. The material seeded in tetrathionate broth the first day was transferred to brilliant green nutrient agar, and tubes of fresh tetrathionate broth were seeded with one loopful which, on the succeeding day, was streaked on nutrient agar. Brilliant green was added to the tetrathionate broth at the rate of 1 : 100,000 in several instances. Plain nutrient agar was used along with the bril-

liant green agar to permit the growth of other organisms present.

All cultures were examined microscopically, and all rod-shaped organisms were placed in four differential carbohydrate broth mediums, dextrose, maltrose, lactose, and sucrose, each containing phenol red as indicator.

No *S. pullorum* was recovered, but the *S. gallinarum* was isolated from 5 of the 12 birds cultured, *i. e.*, from the ovaries of 2 hens, from the liver of 1 hen, from the heart of 1 tom, and from the testes of 1 tom.

This study suggests that fowl typhoid of turkeys may be transmitted through the egg and that the organism is easily isolated from the reproductive system of both males and females. If the disease can be introduced into a flock in this way, it could cause greater death loss than pullorum. Hence, it is possible that fowl typhoid should be regarded equally as dangerous as pullorum, and the two should be given equal recognition when control methods are being planned. It also suggests a possible explanation for outbreaks of fowl typhoid that occur in the brooder house or on the range where it has seemed impossible to account for its introduction from an outside source.

SUMMARY

Fertile eggs from 17 turkey hens, reactors to the pullorum test, were incubated, the embryo killed by chilling, reincubated, and then examined bacteriologically. One egg, out of 374 examined, yielded *Shigella gallinarum*; *Salmonella pullorum* was not recovered. *S. gallinarum* was not recovered from cultures of the organs of any of the birds used in the egg study, but *S. pullorum* was isolated from the ovaries of 2 reacting hens.

Twelve hens, reactors to the tube agglutination test for pullorum, were submitted for examination for *S. pullorum*. No pullorum organisms were recovered from these birds, but the fowl typhoid organism was isolated from 5 of the 12 birds cultured.

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Transmission of the Virus of Newcastle Disease to the Syrian Hamster

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THE WORK of Kraneveld and Masoetion quoted by Beaudette showed the virus of Newcastle disease [pneumoencephalitis] was not established in white mice. Thus far, the Newcastle virus has not grown successfully in hosts other than fowl.[1, 2]

The California strain of Newcastle virus used for the present study was obtained from the Bureau of Animal Industry, Department of Agriculture, Washington, where it has been used for routine neutralization tests with serums from suspected cases. In 1944 C. A. Brandly, while pursuing experimental work at Harvard University on a war research project concerned with poultry diseases, isolated the virus from lung tissue collected by J. R. Beach of the University of California from a chicken naturally affected with pneumoencephalitis. The virus had been passed through six series of embryonated chicken eggs when it was received by the Bureau of Animal Industry, where it was put through ten more such passages. Although the virus was in the sixteenth embryo passage when it was received by our laboratory, it was subjected to six additional such passages before the hamster trials were begun.

One tenth of 1 cc. of infected allantoic fluid from the twenty-second embryo passage, which titered 10^{-4}, was injected intracerebrally into each of 3 hamsters, approximately 4 weeks of age. For two days after injection, these hamsters, as well as the hamsters of successive passages, appeared normal. Symptoms of irritability and malaise appeared three to six days after inoculation. In those cases in which paralysis occurred, the forelegs became affected first in one to two days after the first symptoms appeared. Paralysis progressed rapidly so that within several hours there was complete paralysis of all of the legs and, in from three to six hours, the animals became moribund but survived for twelve to eighteen hours longer. During the moribund period, the animals showed labored breathing and moved only slightly when disturbed.

TABLE 1—Hamster Passages of a Strain of California Newcastle Virus

Passage no.	No. animals inoculated*	Animals paralyzed, moribund, or dead		No. days after inoculation paralysis occurred
		No.	%	
1	3	2	66.6	4 - 4
2	3	1	33.3	5
3	3	3	100.0	3 - 5 - 5
4	3	2	25.0	5 - 9
5	5	2	40.0	7 - 8
6	3	1	33.3	7
7	4	1	25.0	6
8	4	4	100.0	3 - 3 - 4 - 5
9	3	3	100.0	3 - 4 - 4
10	4	4	100.0	3 - 3 - 3 - 4
11	3	3	100.0	3 - 3 - 4
12	3	3	100.0	3 - 3 - 4

*Intracerebrally.

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The authors wish to express appreciation for the coöperation and valuable assistance of Oswald L. Osteen and Wayne A. Anderson of the Bureau of Animal Industry, Department of Agriculture, Washington, D. C., in the instruction in virus neutralization techniques.

The hamsters from which brain material was used for passage were sacrificed while moribund. At autopsy of the sacrificed animals, the spleen, heart, kidney, lung, and brain showed no gross lesions. A 10 per cent brain suspension used for passage was prepared by grinding the brain tissue in a mortar with alundum and diluting with physiologic saline. Eight hundredths to 0.1 cc. of the brain suspension was passed serially through 12 passages in hamsters of an average of about 40 Gm. in weight. Table 1 indicates the results obtained.

bryonated eggs. The test hosts for neutralization were White Leghorn eggs incubated ten to twelve days. Positive, chicken convalescent serum neutralized the hamster virus in both neutralization tests while normal chicken serum did not as shown in table 2.

SUMMARY

A California strain of Newcastle virus was successfully carried serially through 12 Syrian hamster brain passages. The disease in hamsters was characterized by symptoms of irritability and malaise, followed by paralysis, with labored breathing during the moribund period. Hamsters typically affected did not recover. Approximately one third of the injected hamsters failed to show symptoms. Surviving hamster serum contained neutralizing antibodies in one instance, that is, from the fifth passage. Convalescent chicken serum neutralized the Newcastle virus from hamster passage while normal chicken serum failed to neutralize this virus. Further

TABLE 2—Comparative Neutralization Test Results

	Virus dilutions								
	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}
California strain of Newcastle virus									
Specific antiserum Chicken		1/4	0/4	0/4	0/4				
Normal serum Chicken					4/4	4/4	3/4	1/4	..
Virus titration					4/4	3/4	3/4	1/4	..
Allantoic fluid infected with fifth hamster brain passage suspension									
Specific antiserum Chicken		1/4	0/4	0/4	0/4				
Normal serum Chicken					4/4	4/4	4/4	2/4	1/4
Virus titration 5th hamster passage				4/4	3/4	3/4	4/4	1/4	2/4
Hamster brain suspension of eighth passage									
Specific antiserum Chicken	0/3	0/3	0/3	0/3	0/3				
Normal serum Chicken		2/3	2/3	3/3	2/3	2/3	3/3	2/3	0/3
Virus titration 8th hamster passage		3/3	3/3	2/3	3/3	3/3	2/3	1/3	0/3

Numerator of fraction denotes number of deaths. Denominator of fraction denotes number of embryonated eggs inoculated.

Neutralization tests were employed using hamster brain virus of the fifth and eighth passages. The brain suspension of the fifth passage was injected into the allantoic sac of embryonated eggs. Death of these embryos occurred within seventy-two hours and the allantoic fluid was saved for the neutralization test. The brain suspension of a moribund hamster in the eighth passage was used for a second neutralization test without passing to em-

experiments are being conducted in an effort to prove whether or not the Newcastle virus has been modified sufficiently for the vaccination of chickens.

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Infection and Transmission of Brucella by Ectoparasites

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ON SOME occasions, the transmission of brucellosis to either man or animals cannot be satisfactorily explained. Occasionally, human beings who have not been in contact with infected animals or have not consumed raw milk products became infected. Such cases suggest the possiblity of transmission mechanisms other than the ones already known.

The frequent unexplained appearance of brucellosis in herds of cattle, goats, and swine suggests the probable existence of an efficient vector able to transmit infection to healthy animals. Among the parasites that infest animals, there are ticks, especially *Boöphilus annulatus*, which are found distributed all over the world. Since ticks can parasitize cattle as well as goats and swine, these ectoparasites may serve as a reservoir and as efficient vectors of Brucella organisms in nature.

Butts[1] made studies on the transmission of Brucella with *Dermacentor variabilis* without obtaining positive results. Ruhland and Huddleson[2] reported the experimental infection of one species of cockroach and several species of flies with *Brucella abortus*.

Considering the possibility of infecting ticks and other human ectoparasites with Brucella, the present investigation was performed to establish whether those ectoparasites may play some rôle in the conservation of organisms in nature and in their transmission between animals and man.

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MATERIAL

The following ectoparasites were used: *B. annulatus*, *Amblyomma cajennense*, *Cimex lectularius*, and *Ctenocephalides felis*. Studies of infection of lice with Brucella are now underway, the results of which will be the subject of another paper.

The lots of Boöphilus and Amblyomma were obtained from the northern part of Mexico; bedbugs and fleas were collected in Mexico City. The possibility of previous infection of such ectoparasites was eliminated by permitting half the lot of each species of ticks to feed on normal guinea pigs and the complete lots of bedbugs and fleas to feed on normal white mice. For one month, daily cultures of feces and, every five days, cultures of sections of ectoparasites were performed. The results were negative.

Three guinea pigs and 3 mice were injected subcutaneously with 0.5 cc. each of a living suspension (McFarland scale no. 2) of *Brucella melitensis*, *Br. abortus*, and *Brucella suis*. One species was used for each group of 3 animals. After ten days, the animals were injected by the intraperitoneal route with 0.5 cc. of a concentrated suspension of living organisms (same species) (McFarland scale no. 4). Twenty-four hours after inoculation, ticks, bedbugs, and fleas were put on the infected animals, employing lots of 20 ectoparasites for each species of Brucella. Ticks required from four to eight days to fix, engorge, and drop; bedbugs and fleas fed within a few minutes. Each lot of para-

sites was kept in a sterilized tube. Daily cultures were made of the feces of all the lots, and cultures of transversal sections of ectoparasites were made every five days. Feces were emulsified in a small amount of sterile saline solution and transferred to tryptose agar plates. A serial microscopic study was made of smears from sections of parasites after staining by the Hitz[3] method. The study of histologic sections is continuing.

Once it was confirmed that ectoparasites were infected, they were fed on healthy animals to determine whether infection could be transmitted by biting. Complete studies of hereditary transmission of Brucella organisms were performed only on ticks. Those made on fleas and bedbugs are not yet finished.

RESULTS

The serial cultures of feces showed that all the ectoparasites used may be infected with any of the three species of Brucella. Of 10 *B. annulatus*, kept isolated after the infective meal, 8 eliminated Brucella in the feces on some of the days they were under study; of 10 *A. cajennense*, 6 eliminated organisms; of 10 bedbugs, 5 were found infected, as well as 5 of 10 fleas.

In fleas, daily cultures of the feces were positive from the third day after the infective meal; in bedbugs from the sixth day; and in ticks from the tenth day. In bedbugs and ticks, organisms were still present after three months. In most examinations, Brucella colonies appeared in pure cultures. The ectoparasites infected with *Br. suis* began eliminating organisms before the others and they showed a higher mortality rate.

In ticks and bedbugs, the smears showed the presence of epithelial cells full of small intracytoplasmic organisms, a picture identical with that described by Francis[4] in ticks and bedbugs infected with *Bacterium tularense*.

The transmission experiments showed that only ticks, Boöphilus as well as Amblyomma, were able to transmit the infection by biting, providing that the meal was not interrupted from the time they fixed until they dropped spontaneously. Of 6 previously normal guinea pigs on which infected ticks fed, 3 developed specific agglutinins and opsonins, and blood and organ cultures were positive for Brucella.

From female infected ticks, 6 lots of eggs were secured and treated with merthiolate solution for half an hour, washed several times in sterile saline solution, and ground with powdered glass and a small amount of sterile saline solution. The emulsion was centrifuged at low speed and the supernatant fluid injected subcutaneously into guinea pigs. Three of the lots produced infection in the guinea pigs.

Experiments are being conducted to establish whether organisms will resist molts of the different stages of the ectoparasites and also to determine if larvae and nymphs from infected females may transmit the infection by biting.

After obtaining the results referred to above, a careful search of naturally infected ectoparasites was made. Ten lots of *C. lectularius* collected in Mexico City from the bedsides of brucellosis patients and five lots of *B. annulatus* collected from a region in the state of Guanajuato where brucellosis is endemic and rather frequent were studied. Each lot was treated with merthiolate solution for an hour and then washed several times in sterile saline solution. They were ground with powdered glass and a small amount of sterile saline solution. The emulsion was centrifuged at low speed and the supernatant fluid injected subcutaneously into guinea pigs. The animals were bled every eight days for serologic and opsonic tests and blood cultures, according to methods described elsewhere[5, 6]. Two lots of *C. lectularius* were found to be infected with *Br. melitensis* and one lot of *B. annulatus* was infected with *Br. abortus*. Two lots of ticks produced fatal infections, with diffuse and hemorrhagic lesions, in guinea pigs within seventy-two hours. The causative agent which was not Brucella has not been identified.

The classification of the Brucella cultures isolated was performed following the usual methods, including the atmosphere of isolation, H_2S production, bacteriostatic action of dyes, and serologic tests.

DISCUSSION

The above experiments showed that the epidemiologic field of brucellosis is much more complex than is generally believed. The ectoparasites that were found naturally infected with Brucella probably play an important rôle in transmitting brucellosis between animals and human beings as well as conserving the organisms in nature. The finding of naturally infected ticks justifies the complete cleaning of animals besides separating infected cattle to effectively control bovine brucellosis. The egg transmission of organisms complicates the problem of control, since, as in tick-borne rickettsioses, the factors related to the biology of vectors are so varied that they hardly could be totally eliminated.

It was demostrated that *A. cajennense* can be infected with Brucella and is able to transmit the infection by biting and through eggs. It is possible that other varieties of ticks that parasitize infected cattle may also become infected.

Even though bedbugs do not transmit the infection by biting, they eliminate Brucella in the feces for a long time. They may infect human beings by direct contact through the skin or mucous membranes or by the digestive tract. Contaminated feces may also infect through the respiratory tract which, in the laboratory, constitutes one of the most frequent ways of contracting brucellosis.

Infected fleas eliminate great quantities of Brucella in the feces and, as they usually defecate close to the place where they bite, a person could easily infect himself upon scratching, or through the mucous membranes of the digestive or respiratory tracts.

In relation to the facts referred to above, brucellosis patients should be considered a possible source of infection and should be freed of ectoparasites.

SUMMARY

Experiments of infecting ticks, bedbugs, and fleas with Brucella are reported. All of the ectoparasites studied were susceptible to infection with the three species, *Brucella melitensis, Brucella abortus,* and *Brucella suis, Br. suis* being the most infective for all. Fleas were the first to eliminate Brucella in the feces after the infective meal. Ticks and bedbugs were still eliminating Brucella in the feces after three months of observation, yielding, in most cultures, a confluent growth of Brucella free of contamination. The cultures of sections of all infected ectoparasites yielded a confluent growth of Brucella.

Ticks were the only parasites found able to transmit infection by biting if they were not interrupted while feeding. Ticks were also able to transmit organisms to the eggs and larvae.

Smears from infected ectoparasites showed epithelial cells full of small organisms with a morphology typical of Brucella. The microscopic study and cultures of non-infected ectoparasites were negative.

The isolation of one strain of *Br. abortus* from *Boöphilus annulatus* and two strains of *Br. melitensis* from *Cimex lectularius,* collected in the field, is reported.

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